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Lactic acid bacteria fermentation of wort as a tool to add functionality in malting, brewing and novel beverages

Thesis presented by
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Declaration

I hereby declare that this thesis is my own work and effort, and that it has not been submitted for another degree, neither at the National University Ireland, Cork nor elsewhere. Where other sources of information have been used, they have been acknowledged.

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Signature

Date: 07/04/2017

Abbreviations

°P,	Degree Plato
3-PLA,	3-phenyllactic acid
α-AL,	α-acetolactate
AFW,	Acid fermented wort
ALAB,	Amylolytic lactic acid bacteria
BA,	Biological acidification
BC,	Buffering capacity
CA,	Chemical acidification
cfu,	Colony forming unit
CW,	Control wort
CW0.5 + B,	Diluted control wort with buffer
CW + B,	Control wort with buffer
CW + P,	Control wort with protease
D3G,	Deoxynivalenol-3-glucoside
DLG,	Deutsche Landwirtschafts-Gesellschaft
DON,	Deoxynivalenol
ENNs,	Enniatins
EPS,	Exopolysaccharides
FAN,	Free amino nitrogen
FDA,	Food and drug administration
FHB,	*Fusarium* head blight
FID,	Flame ionisation detector
FW,	Fermented wort
GC,	Gas chromatogrphy
GRAS,	Generally regarded as safe
HPLC,	High performance liquid chromatography
IBU,	International bitterness unit
LA,	Lactic acid
LAB,	Lactic acid bacteria
LOD,	Limit of detection
ME,	Malt extract
MEBAK,	Mitteleuropäische Brautechnische Analysenkommision
MIC,	Minimal inhibitory concentration

MRS,	de Man-Rogosa-Sharpe medium
MS,	Mash souring
n.d.,	Not determined
ND,	Not detectable
NIV,	Nivalenol
OW,	Optimised wort
OW+P,	Optimised wort with protease
PFW;	Pasteurised fermented wort
PD,	Potato dextrose
pKa,	Acid dissociation constant
postBWS,	Post-boil wort souring
preBWS,	Pre-boil wort souring
qPCR,	Quantitative polymerase chain reaction
QPS,	Qualified presumption of safety
QuEChERS,	Quick easy cheap effective rugged safe
RID,	Refractive index detector
TSN,	Total soluble nitrogen
TTA,	Total titratable acidity
UV/DAD,	Ultra violet-diode array detector
YC,	Yeast control
ZEA,	Zearalenone

Abstract

Maltsters and brewers are paying increasing attention to lactic acid bacteria (LAB) for novel applications that focus on natural bio-preservation and product diversification. A literature review conducted as part of this thesis revealed that the fermentative metabolism of LAB in cereal-based substrates is the origin of a variety of compounds with preservative attributes and organoleptic characteristics (flavour, texture). Increasing the knowledge of the functional compounds produced by LAB will enable better recognition of the applicative potential of lactic fermentations. This thesis addresses the investigation of several strains of LAB for biopreservation using *in vitro* and *in situ* studies, as well as for acidification of wort to be applied during sour brewing and as novel beverages. The impact of bacterial carboxylic acids, with emphasis on phenolic antifungal compounds, was assessed against *Fusarium culmorum*, a common fungal spoilage organism in malt. The contribution of phenolic acids to the overall fungal inhibition was found to be limited unless combined with lactate and acetate in a low pH environment, suggesting their contribution in a complex, synergistic mechanism. An antifungal ingredient was produced by fermenting wort with *Lactobacillus brevis* R2Δ. When this substrate was applied on barley during steeping and germination, it significantly reduced the amount of fusaria infection, detoxified the substrate and increased the extract yields. To enrich even more bacterial metabolites in wort, an extension of acidification could be achieved by promoting the release of free amino nitrogen through a longer proteolytic rest during mashing or the addition of protease. Distinct sugar preferences, enzymatic capacity and strain-dependent resistance to low pH led to differences in the levels of lactic acid (LA) accumulation. Up to 12.8 g/L LA were achieved in buffered-wort before nutrient-related deficiencies inhibited further acidification. A comparison between acidification methods during production of sour beers showed that, depending on the time point of souring, the beers differed significantly in their processability and flavour profile. Acidification of wort before the boil step was chosen as the preferred technique due to its safer implementation and minimal effect on organoleptic properties. Finally, the sensory profile of fermented, non-alcoholic malt-based beverages revealed that lactic fermentation increased acidity and flavour complexity of these drinks, and reduced compounds that elicit "malty" notes, while increasing the "dairy", "bready", and "honey" character. Forced ageing led to an increase in typical beer off-flavours in all samples, but fermented beverages showed a better organoleptic stability than the unfermented control.

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Chapter 1: Introduction

1.1 Introduction

Lactic acid bacteria (LAB) constitute a diverse group of microorganisms that have been used throughout human history as fermentative agents in food preparation and conservation. They contribute to the organoleptic and nutritional improvement of fermented products as well as to the inhibition of spoilage microorganisms (Leroy et al., 2004). The inability of LAB to synthesize several growth factors, particulary essential amino acids, restricts their occurrence to nutrient-rich substrates, such as milk, meat and cereals. The capacity of LAB to dominate and acidify cereal substrates opens new possibilities for maltsters and brewers to enrich metabolites with functional application. In this regard, wort is a versatile and highly nutritious medium that can well sustain LAB growth and their metabolic output.

Malting, or the controlled germination of cereal grains, is a complex biological and biochemical process, and the microbial communities that naturally colonise the grain surface can influence the performance in terms of processability and safety of the final malts (Laitila et al., 2007). The favourable processing conditions found during steeping and germination can encourage the growth of spoilage microorganisms (Justé et al., 2011). Problems arise when toxigenic fungi such as *Fusarium* spp. proliferate to levels that cause cereal deterioration, while also posing potentially serious health hazards (Bottalico and Perrone, 2002). The high thermostability of *Fusarium* mycotoxins explains their survival along the production chain from grain to malt and finally beer (Lancova et al., 2008; Wolf-Hall, 2007). The increasing interest in replacing traditional preservation methods with natural, clean-label technologies, has encouraged the search for biological alternatives as antifungal agents (Pawlowska et al., 2012). The application of LAB starter cultures during the early stages of malting has been found to significantly antagonise spoilage contaminants (Laitila et al., 2002; Laitila et al., 2006; Lefyedi and Taylor, 2007). and reduce mycotoxin levels, e.g. DON and ZEA (Oliveria et al., 2015; Peyer et al., 2017). Wort can be used as a readily available, food-grade and cheap substrate by maltsters for the enrichment of LAB and the antifungal compounds that they produce (Oliveria et al., 2014; Peyer et al., 2016a). A complex, synergistic mechanism between these metabolites has been suggested to be at the core of the overall antifungal effect of LAB (Axel et al., 2015; Schwenninger et al., 2008). In addition, organic acids and other unidentified compounds exert a phytotoxic effect on germinating barley, allowing the maltster to control the extent of malting losses (Mauch et al., 2011; Schehl et al., 2007).

Lactic acid (LA) is a versatile acidulant, flavour enhancer and preservative in the food industry (Abdel-Rahman et al., 2013). Acidified wort has been used to control pH during brewing operations (Narziss, 1984), and recently, the growing popularity of sour beers in the craft-brewing sector calls for a more in-depth knowledge on bacterial acidification of wort. During batch fermentation, LAB growth and metabolism are increasingly self-inhibited by the low pH, which limits the extent to which LA can be accumulated. Different strategies could be adopted to extend the time of LA production, such as acid neutralisation, improvement of buffering capacity or nutrient addition (Hofvendahl and Hahn-Hägerdal, 2000). Ultimately, the technology chosen should be easy to operate, cost-efficient, and comply with the legislative framework to which the brewery adheres. Traditional sour beer styles, e.g. *Lambics,* are the result of a widely diverse inoculum that plays a role in fermentation and maturation, which can last between 1 to 3 years. Even though the long ageing process is crucial in delivering the distinctive flavour profile of these beers, brewers have also looked into simpler and faster ways to produce acidic beers (Tonsmeire, 2014). The prevailing alternative consists in applying lactic acid cultures to acidify a batch of mash and/or wort for 1 to 3 days, followed by an alcoholic fermentation by yeasts. Depending on the bacterial inoculum, the substrate and/or other fermentation control variables (e.g. duration, temperature, …), the brewer could expect significant differences in the processability and in the overall quality of the final beers.

In the last few decades, the growing awareness of the impact of nutrition-related health problems on quality of life has led to increasing interest in added-value products. Cereal-based beverages fermented by LAB have the potential to fill this market gap. They can be promoted thanks to the intrinsic content of health-related metabolites in wort, e.g. minerals, vitamins, and phytochemicals (Katina et al., 2007). Moreover, lactic fermentation can enrich the substrate with functional metabolites (Waters et al., 2013; Zannini et al., 2013), reduce anti-nutritive factors (Singh et al., 2015) and improve the organoleptic profile (Nsogning Dongmo et al., 2016; Peyer et al., 2016b). LAB are already familiar to the consumer as probiotic cultures in dairy products, which would favour their introduction and bodes well for the acceptance of such wort-based products. Regarding the flavour profile, this can be improved by conducting a targeted selection of the starter culture according to the organoleptic profile that they impart. Different metabolic capacities give rise to an array of flavour and aroma profiles which

are species-, substrate- and process-dependent. Common flavour-active metabolites from LAB are lactic and acetic acid, diacetyl, acetoin, and acetaldehyde.

The main objective of this thesis was to evaluate the functional properties of a barley malt substrate (wort) after fermentation by lactic acid bacteria, for application in malting and brewing, and novel non-alcoholic beverages Figure 1(Figure 1).

Firstly, wort was assessed as a substrate for the enrichment of antifungal carboxylic acids (organic and phenolic acids) during lactic fermentation, and its subsequent capacity to inhibit the *in vitro* growth of the spoilage fungus *Fusarium culmorum* (**Chapter 3**). Fermented wort was further applied as an antifungal ingredient to control the indigenous microflora of barley grains and to enhance technological performance during malting (**Chapter 4**). To improve lactic acid production during fermentation, the buffering capacity of wort was increased by combining different mashing profiles with addition of protease and compared to the addition of exogenous buffering compounds (**Chapter 5**). A comparison of LAB acidification performed at different stages along the brewing process was done to evaluate the advantages and the shortcomings on the processability and final quality attributes of sour beers (**Chapter 6**). Finally, the suitability of wort as a base for a novel, non-alcoholic beverage was studied by applying different LAB cultures and assessing beverage physicochemical attributes, sensorial qualities and flavour stability (**Chapter 7**).

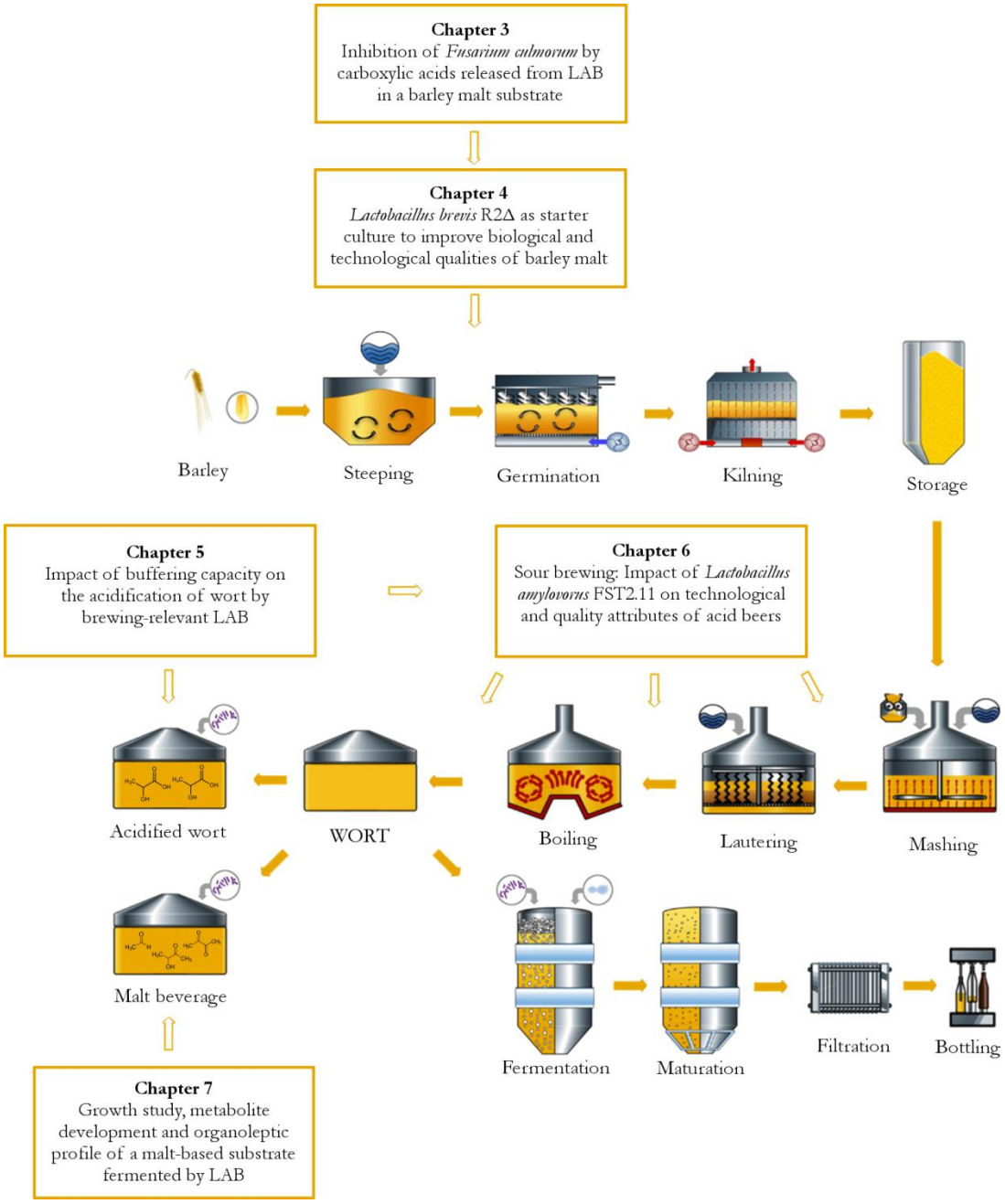

Figure 1. Overview of the malting and brewing processes and their association with the chapters of the thesis.

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Chapter 2: Literature review - Metabolic properties of lactic acid bacteria for use in malting, brewing and beverage production

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2.1 Abstract

Lactic acid bacteria (LAB) are used around the world to improve the preservation, organoleptic characteristic and nutritional value of a large variety of food and beverage products. In malting, LAB have been primarily applied to control the indigenous microbial population that naturally colonise the cereal surface. Due to the release of antimicrobial metabolites and their active role as competitors for nutrients, LAB can successfully improve malt safety by hindering spoilage of aerobic heterotrophic bacteria and filamentous fungi. At the same time, the mild acidification of lactic acid has a beneficial effect on malt quality and processing yields, such as increasing the soluble extract content or reducing wort viscosity while improving wort filtration. LAB have also been recognised as necessary agents to naturally acidify mash and/or wort during brewing operations as well as for the production of acidic beers, e.g. *Berliner Weisse* or *Lambics*. To increase the yields of LA during fermentation of the wort substrate, acidity needs to be counteracted to prevent early self-inhibition of the strains. Different methods can be adopted to do this, such as adding neutralising chemical agents or buffer compounds. Moreover, the nutritional content of the substrate should be aligned according to the strain-dependent auxotrophies of the starter culture. Increasingly, cereal-based beverages have been explored as *functional* and *probiotic* foods because of their nutritious and health-promoting properties, e.g. soluble fibres and phytoestrogens. LAB can be used as natural agents to add functionality and improve the low organoleptic attributes of raw cereals. Starter cultures that are able to release desired flavour compounds or to positively influence the food structure *in situ* have been successfully used to enhance the palatability of cereal beverages and to overcome the need for additives, i.e. flavourings, enzymes or thickeners.

2.2 Re-discovering fermentation

As a way to keep pace with the trends of *sustainability, naturalness* and *wellness*, food companies have reintroduced practices that favour low-processing and low-energy impacts (Athapol et al., 2014). Fermentation has long been used as a way to naturally improve and fortify a substrate, without the need for additives or preservatives (Hugenholtz, 2013). In the interest of ease of control and reproducibility of final product qualities, the industry employs defined starter cultures. Lactic acid bacteria (LAB) have been traditionally applied in many food substrates e.g. dairy, meat, vegetable, and cereal substrates, as well as being part of their indigenous microflora (Holzapfel, 1997), and many strains have been granted GRAS (Generally Recognised as Safe) status. This group of bacteria share several morphological, metabolic, and physiological characteristics. The general description of the bacteria included in the group is Gram-positive, catalase negative, non-motile, non-respiring and non-spore forming cocci or rods. From a food-technology point of view, the following genera are most relevant: *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella*. LAB produce lactic acid as the major end-product during the fermentation of carbohydrates, and their tolerance to acidic environments gives them the competitive advantage over other bacteria (Dicks et al., 1995). In the context of malting, brewing and novel beverages, the ability to release acids as well as other functional metabolites can be used to improve the quality and safety of malting barley (**Chapter 3 and 4**), while acidified wort (**Chapter 5**) constitutes the main ingredient in the production of soured, alcoholic (**Chapter 6**) and non-alcoholic (**Chapter 7**) cereal-based beverages.

2.3 LAB in malting

2.3.1 Natural microflora of brewing cereals

The microbial community that naturally colonizes malting grains consists of numerous species of Gram-negative and –positive bacteria, viruses, yeasts, filamentous fungi, slime moulds and protozoa (Justé et al., 2011). The composition and magnitude of this microbiota are mainly determined by the conditions under which the crop was grown on the field and the post-harvest history of the grain (Flannigan, 1996). Bacteria normally dominate the culturable microflora of pre-harvest barley, followed by yeasts

and filamentous fungi (Angelino and Bol, 1990; Flannigan, 2003). During malting, grains are placed under conditions that favour microbial growth. The first stage, steeping, is a critical step where microbial propagation can occur. Steeping consists of a successive series of wet stands, where the grains are soaked under water, and air rests, where the grains are kept in aerobic conditions under high moisture levels. During the following several days, germination of the grains results in synthesis of enzymes and kernel modification under strictly controlled conditions (temperature, moisture, aeration). For barley, steeping and germination steps are typically carried at temperatures between 14 and 16°C. During the third stage, kilning, the green malt is dried to reach moisture levels of 3 to 4% and germination is halted (Kunze, 2004).

During malting, the presence of leaching nutrients on the grain surface and the beneficial process conditions (over 95% relative humidity and mild temperatures) can enable the growth of the indigenous microflora. Depending on the prevailing microbial populations and their interactions with the germinating kernels, the safety and quality of the malt can differ highly (Justé et al., 2011; Laitila et al., 2007a). Indigenous microorganisms have been recognised as important contributors to malt processability and enzymatic potential, linked to both beneficial and harmful effects (Bokulich and Bamforth, 2013; Justé et al., 2011; Vaughan et al., 2005). A particular source of spoilage and safety concern for the malting and brewing industry, as well as for consumers, are representatives belonging to the group of filamentous fungi. Fungal spoilage has been identified as being responsible for the loss of 5 to 10% of the world's food production (Pitt and Hocking, 2009). Among this group, much research efforts have focused on reducing contamination by *Fusarium* fungi. Many species of this genera are prolific mycotoxin producers, and *F. graminearum*, *F. avenaceum* and *F. culmorum* are predominantly responsible for *Fusarium* head blight (FHB) in wheat and other small-grain cereals (Bottalico and Perrone, 2002). In addition, some species can induce *gushing*, a spontaneous over-foaming of beer correlated to specific fungal compounds i.e. hydrophobins (Sarlin et al., 2007). *Fusarium* spp. can rapidly grow during the first hours of steeping, even from low initial levels of infection (Noots et al., 1999) and can lead to complications such as lower germinative energy, increased water sensitivity and higher malt losses (Oliveira et al., 2013).

2.3.2 Impact of LAB starter cultures on malt quality

Consumer aversion to synthetic chemical preservatives has led food manufacturers to explore new processing and preservation technologies (Zink, 1997). Competitive microbial inhibition by harmless biological agents has been evaluated as a way to control growth of both spoilage and pathogenic microorganisms in malting. Starter cultures of both fungal and bacterial nature have been applied during malting to control microbial infection and/or improve specific malt characteristics, e.g. *Wickerhamomyces anomalus (*synonym *Pichia anomala)* and *Lb. plantarum* (Laitila et al., 2007b), *Geotrichum candidum* (Boivin and Malanda, 1997; Foszczynska et al., 2004), *Pythium oligandrum* (Rezanina, 2014), *Aspergillus giganteus* (Barakat et al., 2010) or *Erwinia herbicola* (Van Campenhout et al., 1998). Among the suitable candidates, LAB (Table 1) have often been the preferred choice as starter culture owing to the production of antimicrobials and hydrolytic enzymes and their consequences on both the biological and process-technical attributes of malting grains (Lowe and Arendt, 2004; Rouse and van Sinderen, 2008).

According to Table 1, AB application on grains during malting can reduce both fungal and aerobic bacterial contamination, while promoting the yeast population. The alterations in the grain microflora can, in turn, influence brewing-relevant quality factors. Changes in attributes such as viscosity, lautering rate, β-glucan levels, nitrogenous compounds (free amino nitrogen, total soluble nitrogen), are the results of a combination of enhanced endogenous enzymatic activities due to acidification (Hattingh et al., 2014; Lowe et al., 2005a), additional bacterial enzymatic activities (Laitila et al., 2006; Lowe et al., 2005a; Lowe et al., 2006), and/or the reduction of the indigenous bacterial microflora (Laitila et al., 2006).

Table 1. Studies employing LAB as biological agents for safety and quality of grains during malting.

LAB species	**Grain**	**Culture(s) application**	**Microbiological changes ***	**Technological changes ***	**Reference**
Lb. plantarum VTT E-78706 (E76)	Barley	In steeping water (cells and spent MRS medium)	↓ Indigenous microflora (depending on contamination level and type)	-	(1)
Lb. plantarum VTT E-78076 (E76), *P. pentosaceus* VTT E-90390 (E390)	Barley	In steeping water (cells and spent MRS medium)	↑ Yeast ↓ Bacteria and *Fusarium* spp.	↑ β-glucanase and xylanase, lautering efficiency ↓ Viscosity, β-glucan	(2)
Lb. amylovorus FST 1.1, *Weissella confusa* FST 1.31	Barley with added *F. culmorum* TMW 4.0754 spores and/or mycelia	In steeping water (cells and spent MRS4 medium)	-	↑ Viscosity ↓ Filtration rate, extract, fermentability, TSN, FAN	(3)
Lb. plantarum (VLS#3, G.M.L.L. 100, I5GR, AB1, AB16b, AB24, AB26, AB31, AB46)	Barley	In steeping water and sprayed at germination (cells and spent MRS5 medium)	-	↑ β-glucan ↓ Malting losses, friability, enzymatic content	(4)
Lb. amylovorus DSM19280, *Lb. reuteri* R29	Pre-infected barley (*F. culmorum* TMW 4.2043)	In steeping water (cell-free supernatant based on wort substrate (3°P, 6°P, 12°P)	↓ *F. culmorum* mycelia (- 23%), mycotoxins DON (- 83%)	↑ Modification, colours, extract yields ↓ pH	(5)
Lb. plantarum L9, *P. pentosaceus* L5	Sorghum	In steeping water (cells with spent MRS or PA	↓ Moulds (- 1 to 2 log) and coliforms (- 3 log)	No changes on diastatic power	(6)

		media)			
Lb. amylovorus FST 1.1, *Lb. plantarum* TMW 1.46, *Lb. amylolyticus* TMW 1.268	Barley	In steeping water (cells and spent mMRS4 medium)	-	↑ β-glucanase, lautering efficiency, TSN ↓ Friability, viscosity	(7)
Lb. plantarum	Barley	In steeping water (combination of LAB with cfs of *Aspergillus*, *Rhizopus* and *Trichoderma*)	-	↑ FAN ↓ β -glucan	(8)
Lb. plantarum 15GR, *Lb. plantarum* TMW1.460	Barley	In steeping water (cells and spent MRS medium	-	↓ Malting losses (- 50%)	(9)
Lb. brevis R2Δ	Barley	In steeping water and sprayed at germination (cells, cells + wort medium, wort medium)	↑ Yeast ↓ Aerobic bacteria, *Fusarium* spp. infection (- 90%)	↑ Extract yield ↓ Malting loss, friability, α-amylase, mash pH	(10)

* Increase and decrease of single attributes are given by the arrows ↑ and ↓, respectively.

Reference: (1) Laitila et al., 2002; (2) Laitila et al., 2006; (3) Lowe et al., 2006; (4) Mauch et al., 2011a; (5) Oliveira et al., 2015; (6) Lefyedi and Taylor, 2007); (7) Lowe et al., 2005a; (8) Hattingh et al., 2014; (9) Schehl et al., 2007; (10) Peyer et al., 2017.

Repeatedly, the application of LAB cultures during malting has shown a reduction of malting losses. *Malting loss* can be defined as the loss of grain substances due to the respiration, leaching of grain substances in steeping liquid and rootlet removal during malting, which accounts for 3.5-5%, 0.5-1.5% and 2.5-4.0% loss of the dry based kernel weight, respectively (Briggs et al., 1981). The main metabolites released by LAB can exert a phytotoxic action on the germinating barley when exceeding critical concentrations, thus representing another possible tool to improve malting efficiency and yields (Lynch, 1980). Schehl et al. (2007) were the first to report the significant

impact of LAB application on rootlet growth. *Lb. plantarum* 15GR was found to reduce malting losses by 50% during pilot scale malting of barley, performing significantly better than the chemical rootlet inhibitor potassium bromate whose use is currently not allowed under European legislation. Good malt quality was maintained, and no negative effects were found on the produced wort. The authors reported that lactic acid and other unidentified compound(s) were responsible for the inhibition of rootlet growth. A recent work by Mauch et al. (2011a) successfully reported a 70% reduction of malting loss due to the addition of *Lb. plantarum* starter cultures directly after steeping of raw barley. Inhibition of the metabolic activity of the kernel also caused reduced modification of the barley endosperm, indicating that organic acids and potentially other substances interfere with the synthesis of these enzymes in the aleurone layer. This was seen by the reduction of overall malt modification. Apart from the α-amino nitrogen levels that could not be corrected to optimal levels, the wort and final beer produced from this malt were of acceptable quality. However, the application of specific temperature rests during mashing was required to compensate for the suboptimal modification (Mauch et al., 2011b).

2.3.3 Antifungal LAB during malting

Acidification of the substrate by organic acids has long been regarded as the main component responsible for inhibition of spoilage organisms (Schnürer and Magnusson, 2005). However, while the majority of studies concerned with the bioprotection of malt examined the application of a cell-free spent medium for reasons of cross-contamination, others have emphasised the advantages of applying the entire fermentation culture (cells and spent medium) in order to benefit from both the antimicrobial compounds released and the *in situ* action of the strains (Laitila et al., 2002). This can further add to the inhibitory mechanisms including competition for nutrients, space and exclusion of the pathogen from entry sites in the matrix (Pawlowska et al., 2012).

Recent investigations have shown that antifungal metabolites, other than organic acids, might be involved in the bioprotection of cereal-based food products. Several secondary metabolites of LAB have demonstrated antifungal activity, including, acetoin, carbon dioxide, diacetyl, hydrogen peroxide, caproic acid, 3-hydroxy fatty acids, phenolic acids, cyclic dipeptides, reuterin, fungicins and other proteinaceous compounds (Rouse and

van Sinderen, 2008). A comprehensive overview of LAB antifungal compounds, their mechanisms of action and some applications of such antifungal LAB was recently reviewed by Crowley et al. (2013).

Among the compounds produced, phenolic acids have recently garnered interest, as several of these low-molecular weight compounds (e.g. 3-phenyllactic acid (3-PLA) and benzoic acid) have been shown to retard or terminate fungal growth both *in vitro* and *in situ* (Axel et al., 2015; Brosnan et al., 2012; Oliveira et al., 2014; Svanström et al., 2013). Firstly recognised by Mandal et al. (2007) as compounds released by *P. acidilactici* in fermented meat, phenolic compounds were further investigated as biopreservatives during food processing, with applications in cereal-based substrates during sourdough fermentation (Axel et al., 2015; Ryan et al., 2009) as well as during barley malting (Oliveira et al., 2014; Peyer et al., 2016).

While 3-PLA has been the most studied of the antifungal phenolic compounds (Mu et al., 2012), little research has considered the broader spectrum of phenolic acids released by LAB. In this regard, Oliveira et al. (2015) found thirteen phenolic compounds after fermentation of wort by *Lb. reuteri* R29 and seven by *Lb. amylovorus* DSM19280, confirming that the type of antifungal compounds varied significantly depending on the fermenting LAB. Among them, 3-PLA, OH-PLA and benzoic acid were present at significant concentrations. Axel et al. (2015) reported the higher content of 3-PLA, OH-PLA, phloretic acid, and hydroferulic acid in quinoa sourdough fermented with the antifungal strain *Lb. amylovorus* DSM19280 when compared to a non-antifungal strain. This strain produced sourdoughs with an extension of the mould-free shelf life by 4 days compared to the non-acidified control. The presence of antifungal-active phenolic acids (0.1-360 mg/kg) were also reported in freeze-dried sourdoughs, and contributed to an extended shelf life of wheat bread by 6 days (Axel et al., 2016). The vast majority of the phenolic metabolites reported in these studies were released at levels below their minimum inhibitory concentration (MIC). When adding the same levels of synthetic phenolic acids to bread, this did only result in an extension of shelf life when the dough was further chemically acidified with organic acids (+25% days). Therefore, it is suggested that an additive and synergistic effect between organic acids and phenolic compounds is responsible for the overall antifungal capacity rather than the individual compounds (Axel et al., 2015; Brosnan et al., 2012; Lavermicocca, 2003; Niku-Paavola et al., 1999; Peyer et al., 2016).

Antifungal compounds produced by LAB have been generally been enriched using a synthetic, nutritional broth, e.g. de Man-Rogosa-Sharpe (MRS) medium. This has the advantage of providing the necessary essential nutrients and high buffering capacity required to sustain LAB growth and inhibitor release during fermentation. The main drawbacks of nutritional broths are the prohibitive costs associated with large-scale commercial application and the presence of unapproved (non-food grade) ingredients (Laitila et al., 2004). For this reason, research has been pursuing the use of unconventional raw materials as substrates (Pawlowska et al., 2012; Pitt and Hocking, 2009). The attractiveness of a food-grade ingredient relies on its direct application to the barley surface while avoiding expensive down-stream costs of antimicrobial purification. Cereal-based substrates such as wort have been shown to accommodate the high nutritional requirements of LAB cultures while enriching antifungal compounds. Moreover, single synthetic ingredients could also be substituted with natural alternatives. Laitila et al. (2004) found that malt sprout, a by-product of the malting industry, could effectively replace peptone as the main nitrogen source, achieving comparable growth of *Lb. plantarum* cultures to that of synthetic medium.

2.3.4 Detoxification of malting grains

Mycotoxins are products of the secondary metabolism of filamentous fungi (micromycetes) and are known for their toxic effects on humans and animals, causing a range of acute and chronic symptoms (Beláková et al., 2011). Mycotoxins are generally stable and are neither degraded nor removed during most food processing operations, thus contaminating the final product (Bullerman and Bianchini, 2007). They are mainly produced in the field and therefore are already present on the raw grains prior to downstream processing. However, malting can further enhance both the fungal growth and the mycotoxin load, which can exceed the initial level of contamination (Lancova et al., 2008). The toxin deoxynivalenol (DON) was found to increase 8-fold during malting of barley grains infected with *F. culmorum*, with the highest accumulation during kilning (from 70 μg/kg to 348 μg/kg) (Oliveira et al., 2012). Numerous studies have shown that defined LAB strains can be successfully employed *in vitro* to detoxify mycotoxins released by *Fusarium* spp. (T-2 toxin, DON, nivalenol (NIV), zearalenone (ZEA)) (Hathout and Aly, 2014). Hathout and Aly (2014) have reviewed the current biological methods for detoxification of food and feed *in vitro* and *in vivo*.

El-Nezami et al. (2002a) found that the ability to detoxify a substrate using LAB cultures is highly strain-specific. The authors found that removal of seven mycotoxins belonging to the trichothecenes family varied significantly depending on the strains and toxins considered. Viable and heat-killed forms of *Lb. rhamnosus* GG were more effective in trapping mycotoxins than *Lb. rhamnosus* LC-705 from liquid media. From a total of 2 μg/mL toxins, this strain was able to bind four of the seven toxins tested, and removal rates varied from 18% to 93%. The detoxifying effect of LAB is mostly attributed to the adsorption of mycotoxins to the peptidoglycan found in the bacterial cell wall (El-Nezami et al., 2004). Niderkorn et al. (2009) further reported that the interaction strength between the fumonisins B1 and B2 (FB1, FB2) and LAB depended on the amino acid composition of the aforementioned peptidoglycans. Recently, Sangsila et al. (2016) showed that the detoxifying capabilities of *Lb. pentosus* increased with higher zearalenone (ZEA) concentrations in the substrate. The best strain bound up to 83% from a solution containing 74.7 μg/mL ZEA toxin. The binding of ZEA and α-zearalenol by lyophilised *Lb. rhamnosus* spp. was found to be immediate after mixing the mycotoxins with the bacteria (55% of the toxin captured), and more adsorption was detected when higher cell concentrations were used (El-Nezami et al., 2002a). Many studies have shown that inactivated LAB cells were more effective in reducing the amount of mycotoxins than the viable counterpart, as found for DON (Franco et al., 2011) and aflatoxin B1 (Peltonen et al., 2011). This is supported by previous studies, which found that heat, acid or enzymatic pre-treatment of LAB might led to increased pore size within the thick peptidoglycan structure of the cell wall, allowing improved mycotoxin adsorption (El-Nezami et al., 2002b; Niderkorn et al., 2006). However, toxins were also decreased when cytosolic preparations of LAB were mixed together, suggesting that other binding mechanisms (e.g. interactions with short chain fatty acids) may play a role in the overall adsorption (Knasmüller et al., 2011; Stidl et al., 2008). The stability of the complexes formed between mycotoxin and LAB can change according to the environmental conditions (Dalié et al., 2010). This has important consequences upon ingestion of the detoxified food substrate. If the complex is characterised by weak binding, e.g. hydrophobic interactions, the mycotoxin may be released from the bacterial surface, becoming bioavailable in the gastrointestinal tract. The recognition of the high binding capacity of non-viable LAB is significant, because the survival rate of these cultures is drastically reduced under the high acidities conditions in the stomach.

2.4 LAB in brewing

2.4.1 Biotechnological process for lactic acid production

LA is a valuable and versatile chemical used in the food industry as a preservative, acidulant, and flavouring (Hofvendahl and Hahn-Hägerdahl, 2000). Recently, the demand for LA has increased significantly because of its role as a monomer in the manufacturing of biomaterials based on polylactic acid (Okano et al., 2010). LAB have been traditionally used for LA production and are still the predominant candidate for its industrial exploitation. The main reasons are the relatively fast process of lactic fermentation, the high yields and the selective enrichment of one of the two LA stereoisomers (L(+) and D(-)) (Von Wright and Axelsson, 2012). LAB can be classified into two groups: *homofermentative* and *heterofermentative*. While the homofermentative LAB convert glucose almost exclusively into lactic acid, the heterofermentative LAB catabolise glucose into ethanol, carbon dioxide, acetic acid as well as lactic acid (Hofvendahl and Hahn-Hägerdahl, 2000; Von Wright and Axelsson, 2012). The homofermentative LAB usually metabolise glucose via the Embden-Meyerhof pathway (i.e. glycolysis). Since glycolysis results only in lactic acid as a major end-product of glucose metabolism, two lactic acid molecules are produced from each molecule of glucose with a yield of more than 0.90 g/g (Smith et al., 1975; Thomas et al., 1979). For efficient industrial production of LA, by-product formation must be avoided, or kept to a minimum. For this reason, only the homofermentative LAB are employed for the commercial production of lactic acid (Hofvendahl and Hahn-Hägerdahl, 2000; Yun et al., 2003).

There have been numerous investigations into improving the efficiency of LA production by LAB from plant biomass. Natural starchy raw materials are, in this respect, promising substrates for LA production, as they are relatively abundant and cheaper than refined sugars (Doran-Peterson et al., 2008). One bottleneck for increasing the cost-efficiency of LA production is owned to the expenses associated with the pre-treatment of these biomasses. These involve physicochemical and/or enzymatic (α-amylase and glucoamylase) steps to saccharify the substrate to glucose (John et al., 2007). However, the direct conversion of starch-rich material to LA is also possible using amylolytic LAB (ALAB) strains. Extracellular amylase activity has been characterised in several lactobacilli, including *Lb. fermentum*, *Lb. plantarum*, *Lb.*

mannihotivorans, *Lb. amylovorus*, and *Lb. gasseri*. Amylolytic lactobacilli are predominantly isolated from fermented meals based on sorghum, rice, millet, maize or cassava (Petrova et al., 2013), supposedly because of the lower β-amylase content in these plants compared to wheat or rye (Gänzle and Follador, 2012). *Lb. amylophilus* GV6, a widely studied ALAB, was capable of very high LA yields (96 g lactic acid produced per 100 g substrate utilised) when fermenting red lentil flour as carbon source and using baker yeast as a nitrogen source (Altaf et al., 2006). The presence of both amylase and pullulanase (debranching enzyme) makes this strain especially efficient in the direct conversion of complex starchy substrates to lactic acid.

Additional costs occur due to the separation and purification of LA after fermentation. LA production via LAB fermentation suffers from end-product inhibition. The addition of neutralising agents, e.g. NaOH, NH_4OH, $Ca(OH)_2$, allows for increasing levels of LA to be produced. Such neutralising agents maintain the pH within a constant range of about 5 to 7, which corresponds to the optimal pH for LA production (Abdel-Rahman et al., 2010). A drawback of adding acid neutralisers is the large amounts of by-product salt (sodium lactate, ammonium lactate and calcium lactate), which has to be converted back into lactic acid before it can be used. Metabolic engineering and traditional strategies of strain mutation and selection have been advanced to alter the acidity resistance properties of an organism. In this regard, *Lb. delbrueckii* has been subjected to mutagenesis to enhance its tolerance to LA, with the mutants producing more LA than the wild-type strain (Demirci and Pometto, 1992). The authors reported that mutant DP3 released LA at a rate that was more than 2-times faster than the wild-type, for a final amount of 77 g/L LA compared with 58 g/L, respectively.

2.4.2 LAB nutritional requirements

LAB have typically complex nutritional requirements due to their limited ability to synthesize their own growth factors (Endo and Dicks, 2014). These can differ considerably, even between strains of the same species. In general, a source of carbohydrates, peptides, amino acids, nucleic acid derivatives, vitamins, minerals, and fatty acid esters is required for sustaining growth (Amrane and Prigent, 1997; Van Niel and Hahn-Hägerdahl, 1999). As reported in Table 2, wort contains a wide range of fermentable nutrients to support LAB growth.

Table 2. Malt wort nutrients (10–12°P) relevant to LAB growth[a].

Carbohydrates (1)	**g/L**
Glucose	5-15
Fructose	1-4
Sucrose	1-5
Maltose	52-60
Maltotriose	13-18
Dextrins	24-42

Vitamins (2, 3, 4)	**µg/L**
Thiamine	150-750
Pyridoxine	150-200
Niacin	1500-2500
Pantothenate	150-250
Biotin	5-10
Riboflavin	300-500
Folic acid	50-100
p-aminobenzoic acid	20-50
Inositol	40000-45000

Organic acids (5)	**mg/L**
Citrate	170
Gluconate	50
Malate	60
Pyruvate	< 10
Lactate	< 10
Succinate	10
Fumarate	10
Oxalate	10
α-ketoglutarate	10

Nitrogenous compounds (2, 6, 7, 8, 9)	**mg/L**
Proteins	138
Polypeptides	155
Peptides and amino acids	400
- *of which free amino acids*	150-230
Ammonia	25-30
Amines	< 10-20
Nucleic acids	280-330

Minerals (5)	**mg/L**
Potassium	550
Sodium	30
Calcium	35
Magnesium	100
Copper	0.1
Iron	0.1
Manganese	0.15
Zinc	0.15
Sulphur	90
Phosphate	575
Chloride	45

Lipids (1)	**mg/L**
Free fatty acids (C4-C10)	0.1
Free fatty acids (C12-C18)	18-26
Mono-/Di-/ and Triglycerides	6.8-10.3
Fatty acid esters	1.2-1.3
Sterol esters	0.1-0.2
Free sterols	0.2-0.4

[a]Data compiled from multiple sources: (1) MacWilliam, 1968; (2) Chen et al., 1973; (3) Graham et al., 1970; (4) Silhankova, 1985; (5) Mandl, 1974; (6) Clapperton, 1971; (7) Jancar et al., 1983; (8) Lie et al., 1974; (9) MEBAK, 2011.

Many simple carbohydrates can be used by LAB as a source of carbon and energy, with glucose being the preferred sugar for most LAB. In obligate homofermentative and facultative heterofermentative lactobacilli, maltose and fructose utilisation generally occurs only after glucose has been depleted (Kandler, 1983). Instead, for the obligate heterofermentative *Lb. sanfranciscensis*, maltose is the preferred carbon source, as this species lacks of the hexokinase enzyme needed for the initial phosphorylation of hexoses (Gänzle and Follador, 2012). Fructose can be used directly as an energy source, but it can also serve as an electron acceptor in obligate heterofermentative LAB, with the production of mannitol and acetate (Axelsson, 1998). Metabolic pathways for disaccharides often allow the catabolism of tri- (e.g. maltotriose) and tetrasaccharides (e.g. maltotetraose). However, since the majority of the catabolising enzymes are found intracellularly, oligosaccharide metabolism in LAB is restricted by the trans-membrane transporters present (Gänzle and Follador, 2012). As mentioned above, long-chain dextrins and/or starch are directly available to strains possessing extracellular amylolyic activity.

LAB have a very limited capacity to synthesize amino acids using inorganic nitrogen sources such as ammonia, and most of them rely on pre-formed amino acids present in the growth medium (Fernandez and Zuniga, 2006). The requirement for single amino acids varies widely among species and strains within the same species. Some species may depend on the presence up to fifteen amino acids, e.g. *Lb. brevis*, whereas other might rely on fewer of them for growth, e.g. *Lb. plantarum* (Barrangou et al., 2011). The mixture of amino acids, peptides, and amides usually stimulates the growth of LAB to a higher extent than if only amino acids are present (Van Niel and Hahn-Hägerdahl, 1999). Some LAB strains have developed proteolytic systems capable of hydrolysing proteins and peptides in order to obtain the required amino acids. In this respect, most dairy starter cultures such as lactococci, used in the manufacture of cheese have proteolytic activities. Many LAB are auxotrophic for both purines and pyrimidines. Nucleotides are obligatory substrates for RNA and DNA synthesis and serve as the main energy donors for cellular processes (Kilstrup et al., 2005). The strain requirement for vitamins varies greatly, but in general, it ranges from one to four vitamins for achieving normal growth, with pantothenic acid, riboflavin, and niacin being essential for the majority of LAB (Wegkamp et al., 2010). Thiamine is especially required when lactobacilli are grown on pentoses (e.g. arabinose, ribose), as it acts as a co-factor for phosphoketolase, an enzyme involved in the pentose phosphate pathway (Hayek and

Ibrahim, 2013). Metal ions are known to serve in membrane transport, as components of molecules or structural complexes and as activators or co-factors of enzymes. The mineral requirement of most strains can be met by supplying a source of potassium (K^+), magnesium (Mg^{2+}), manganese (Mn^{2+}), and phosphate (PO_3^{4-}) ions (MacLeod and Snell, 1947). Traces of Mn^{2+} are essential for the growth and metabolic activity of LAB. These ions play a role in the biological functions of numerous enzymes such as glutamine synthetase, RNA polymerase, and lactate dehydrogenase (Terpstra et al., 2001). Mg^{2+} is another essential metal ion that has been found to stimulate growth and improve survival of LAB (Hebert et al., 2004; Wegkamp et al., 2010). Other than being involved as a co-factor for enzymes, Mg^{2+} acts as an activator of different reactions such as cell division, stabilisation of nucleic acids (DNA, RNA) and peptide hydrolysis (Boyaval, 1989). If, on one side, Mn^{2+} and Mg^{2+} are known to enhance the enzymatic activity, on the other side, heavy metals such as mercury (Hg^{2+}), copper (Cu^{2+}), nickel (Ni^{2+}), zinc (Zn^{2+}) and cobalt (Co^{2+}) can inhibit enzymatic activity (Boyaval, 1989). Recently, potassium ions have been linked with the conduction of long-range electrical signalling within bacterial biofilm communities, with potassium ions triggering the depolarisation across the bacterial ecosystem (Prindle et al., 2015). LAB show no specific iron (Fe^{2+}) requirements and the supplementation of growth media with iron does not stimulate lactobacilli growth (Pandey et al., 1994). There is limited data on the role of fatty acids as nutrients and growth factors for LAB. Most studies have researched fatty acids as inhibitors to LAB (Jenkins and Courtney, 2003), but in small amounts, they have also been to stimulate growth (Partanen et al., 2001; Williams et al., 1947).

Depending on the substrate being fermented, additional ingredients are added to significantly improve LA production. Yeast extract, peptone or corn steep liquor are regularly added to nutrient-poor substrates to improve fermentability (Chiarini et al., 1992). Kotzamanidis et al. (2010) investigated the effects of sucrose, yeast extract and calcium carbonate ($CaCO_3$) on LA production from beet molasses by *Lb. delbrueckii* subsp. *delbrueckii* NCIMB 8130. LA production was significantly affected by both sucrose-yeast extract and sucrose-$CaCO_3$. In particular, sucrose and yeast extract had a linear effect on LA production. The maximum LA concentration (88 g/L) was obtained at a level for sucrose, yeast extract and $CaCO_3$ of 89.93, 45.71 and 59.95 g/L, respectively. Demirci et al. (1992) found that the addition of yeast extract up to 3% in the growth media significantly increased the LA production by *Lb. delbrueckii* subsp.

debrueckii ATCC 9649. MRS medium, which contains a mixture of yeast extract, peptone and meat extract, was found to be superior to yeast extract, which in turn was better than malt extract. Yeast extract alone at high concentration gave higher LA production than yeast extract and peptone in low amounts (Milko et al., 2007) but the opposite resulted when the concentration of yeast extract was kept constant and peptone was increased (Amrane and Prigent, 1997).

2.4.3 Biological acidification in the brewery

LAB play a dual role in the brewing industry. On one side they are negatively associated with the spoilage of beer, leading to textural defects and unwanted off-flavours (Bokulich and Bamforth, 2013), and on the other side, they play an important technological role as processing aids aimed at improving the final product quality (Lowe and Arendt, 2004). Acidification by LAB is a positive tool that can lead to various biochemical, processing-technological and sensory improvements in brewing with standard malt, adjunct grains (Lowe et al., 2004) or for the production of sour beers (Bokulich et al., 2012).

Typically, an average Pilsner malt will give a mash with a pH value of 5.6-5.8 (Kunze, 2010). This pH, however, is higher than the optimum for most of the enzymes involved in the biochemical conversion or hydrolysis of malt storage and structural macromolecules during the mashing process, especially for β-glucanase (4.5-4.8) and carboxypeptidase (4.5-4.6) (Lewis, 1998). Adjusting the pH to a lower level of ca. pH 5.1-5.4 will optimise the overall enzymatic performance during mashing and result in easier processing properties and beneficial effects on the end product.

Breweries that adhere to the German Purity Law and abstain from the application of mineral acids make use of biological acidification (BA) to correct the pH of mash and/or wort (Narziss, 1984). LAB strains commonly used for BA are *Lb. amylolyticus* (Bohak et al., 1998), *Lb. amylovorus* (Vaughan et al., 2005), *Lb. delbrueckii* (Kunze, 2010) and *Pediococcus acidilactici*. Generally, suitable LAB strains should have the following physiological and techno-functional characteristics: homofermentative metabolism, amylolytic activity, and hop sensitivity (Kunze, 2010). Lowe and Arendt (2004) also underlined the importance of the ability of these starter cultures to grow at temperatures up to 48°C in order to give them competitive advantage over other spoilage microorganisms, e.g. butyric acid bacteria and yeast.

As outlined in Figure 2, BA can lead to numerous benefits during brewing processes. A high endogenous β-glucanase activity achieves adequate cytolysis and thus, improved accessibility for the amylases to attack the starch molecules. This can lead to shorter mashing programs (Lewis, 1998). At the same time, the enhanced breakdown of β-glucans reduces mash and wort viscosity and ultimately improves lautering and filtration efficiencies (Kunze, 2010). Better activation of proteolytic enzymes leads to a better break formation during boiling, and finally to a lower risk of protein haze (Pittner and Back, 1995). The action of lipoxygenase reduces as pH gets lower, which leads to a lower breakdown of unsaturated fatty acids and finally, enhanced flavour stability. The beer tends to have lighter colours (Lowe et al., 2005b) with a foam that has finer bubbles and is more stable (Oliver-Daumen et al., 1989). During BA, more positively charged ions are present in the wort, which can react with zinc chelators, leaving more Zn^{2+} free in the wort. This results in positive effects on yeast metabolism, e.g. better diacetyl reduction, and an overall quicker fermentation (Donhauser and Wagner, 1986; Grutzmacher, 1991).

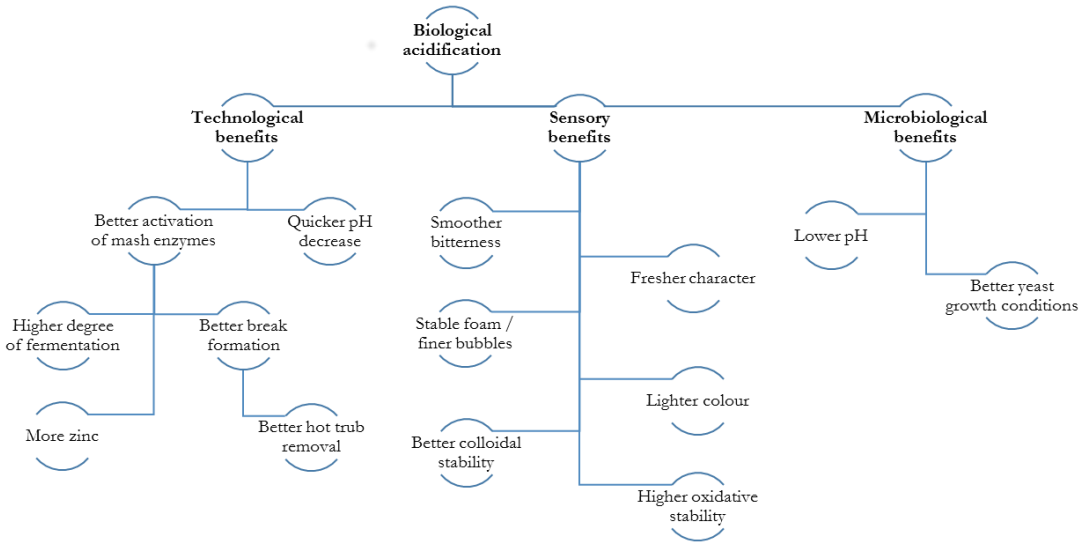

Figure 2. Advantages of biological acidification when applied in the brewing process (adapted from Lowe and Arendt, 2004a; Pittner and Back, 1995; Vriesekoop et al., 2012).

Biological wort acidification is most commonly performed as a batch process (Lowe and Arendt, 2004). Fresh, unhopped wort is mixed with ca. 10% of previously acidified wort containing active LAB. Lactic fermentation takes place between 12 to 48 h at 45-48°C until the desired pH (3.2–3.3) and LA concentration (0.7–1.0%) are reached (Vaughan et al., 2005). BA systems have also been developed as semi-continuous or continuous

processes (Kunze, 2010). The main advantages of a continuous system are attributed to its closed nature, which lowers the risk of microbial contamination, and its suitability for automation when using immobilised LAB (Pittner and Back, 1995).

Normally, about 1% (v/v) of the acidified wort is added during mashing in, 1- 2% (v/v) at the end of mashing for mash acidification, whereas 2% (v/v) are added to the first wort for wort acidification (Kunze, 2010). Only mash acidification, however, does not result in a lower beer pH, as the lower pH during mashing increases the activity of phosphatases and as such liberating buffering phosphates (Vriesekoop et al., 2012). To reach the desired final pH in beer (4.0–4.5), wort acidification is also conducted shortly before the end of the wort boiling process.

2.4.4 LAB during sour brewing

Autochthonous LAB species, together with acetic acid bacteria and/or various yeasts (Van Oevelen et al., 1977; Verachtert and Iserentant, 1995), are responsible for the distinctive sour taste in traditional sour beer styles, e.g. *Berliner Weisse* and *Leipziger Gose* (Germany) (Burberg and Zarnkow, 2009; Kunze, 2010) and *Lambics, Gueuze,* and *Flanders Red Ale* (Belgium) (Van Oevelen et al., 1977; Verachtert and Iserentant, 1995). Strains belonging to the *Lb. brevis* and *Lb. delbrueckii* species have been frequently isolated from *Berliner Weisse* (Preedy, 2009), while *Pediococcus* spp. were the most dominant in *Lambics* and *Gueuze*. Acetic acid in these beers is often produced by other souring microorganisms, e.g. acetic acid bacteria and *Brettanomyces* spp. (Spitaels et al., 2014).

Traditionally, production of *Berliner Weisse* involves the inoculation of yeast and LAB in the order of 4-6 to 1 ratio, respectively (Burberg and Zarnkow, 2009; Schönfeld, 1938). In order to promote LAB and acidification, the temperature of fermentation is raised to 35-45°C (Schönfeld, 1938). However, high levels of LA can compromise the performance of the yeast, leading to low head formation and low carbonation levels (Preedy, 2009). The sharp sourness is imparted by the lactic, acetic and propionic acids, but LAB play only a minor role regarding the aroma formation. The aromatic ester fruitiness, with the presence of high amounts of ethyl lactate and ethyl acetate, derives from species of *Saccharomyces* and *Brettanomyces* (e.g. *bruxellensis*) (Wackerbauer and Methner, 1989).

With no proper boiling step during the traditional production of the *Berliner Weisse* style, a biodiversity of microorganism survives through the brewing process. In the past,

contaminations with slime-forming *Pediococcus* spp. were often reported (Burberg and Zarnkow, 2009). In addition to the undesirable production of high levels of diacetyl ("buttescotch"), *Pedioccocus* can also release exopolysaccharides, which causes an unpleasant increase in the beverage viscosity (Sakamoto and Konings, 2003). Moreover, the fermentation was scarcely controlled and mixed cultures are difficult to keep at a constant ratio over multiple pitching events, causing the end product to lack of consistency. For this reason, commercial *Berliner Weisse* has, more recently, been produced by mixing batches of worts separately fermented by *Saccharomyces cerevisiae* and homofermentative lactobacilli (Wackerbauer and Methner, 1989). The advantages of this split process are that both fermentations can proceed at optimal temperatures for each microorganism; blending allows the achievement of the desired level of sourness, and the lactic stream can be boiled to kill the LAB and avoid further acidification in the final product.

The autochthonous brewery microbiota is the source of the inoculum for well-known Belgian acidic beers. These are generally weakly carbonated products of a spontaneous fermentation process that lasts for one to three years before bottling. Spitaels et al. (2015) identified four LAB isolates belonging to *Lactococcus lactis*, *Leuconostoc citreum* and *Pediococcus pentosaceus* from different sources in the brewery environment of industrially produced *Lambic* beer, while *P. damnosus* was the dominating LAB during fermentation. Thirty-eight LAB isolates belonging to the species *P. damnosus* (previously *P. cerevisiae*) were isolated from wort during fermentation of traditional *Lambic* and *Gueuze* beers. The lactic bacterial population was found to increase after 3 to 4 months, reaching a maximum at the 7th month, after an *Enterobacteriaceae* phase and a main alcoholic fermentation one (Van Oevelen et al., 1977). A similar microbial succession was found during production of spontaneously fermented American coolship ale. Using terminal restriction fragment length polymorphism techniques, Bokulich et al. (2012) tracked the bacterial community structure over 3 years of fermentation. The authors found that after a phase dominated by *Enterobacteriaceae*, *Lactobacillales* became the prevalent population, accounting for from 50-70% (week 4 – 12) to > 90% (week 12) of the total bacterial microflora. The rich biodiversity consisted of species of *Leuconostoc*, *Lactococcus* and *Pediococcus*, with the latter becoming the predominant species within the LAB group by week 4 (> 80%).

With regard to the aroma spectrum, traditional *Berliner Weisse* is similar to the Belgian sour beers. *Lambic* and *Gueuze* are characterised by high contents of lactic and acetic

acid, as well as their esters (ethyl lactate and ethyl acetate) (Van Oevelen et al., 1976). Concentrations between 2.1–3.4 g/L of lactate and 0.5–1.2 g/L of acetate were found in refermented/filtered *Gueuze* samples, which largely exceed the taste thresholds for these acids in beer (0.4 and 0.2 g/L, respectively). *P. damnosus* was also the prevailing LAB present in Belgian red-brown acidic ales at the end of the maturation phase and was most likely responsible for the majority of lactic acid released in these beers (Snauwaert et al., 2016). At the end of maturation, these beers contained lactic and acetic acid concentrations of around 6 g/L and 1.6 g/L, respectively (Maertens, 1997).

2.5 LAB in the production of cereal-based beverages

2.5.1 Cereals as alternative substrates for functional beverages

The domestication of grains during the first agricultural revolution around 10,000 B.C. led to cereals becoming the major source of nutrients and calories for humans throughout the world (Poutanen, 2012). Even though their dietary protein and fat content is inferior compared to other staple foods, e.g. milk or legumes (Chavan et al., 1989), cereals are a most important source of carbohydrate and dietary fibres, and provide essential micronutrients such as minerals, vitamins, and phytochemicals (e.g. phytoestrogens and phenolic compounds) (Katina et al., 2007). LAB often form the natural inoculum, together with fungal strains, of fermented cereal gruels commonly consumed in many rural societies worldwide (Nout, 2009). Fermentation of cereal-based beverages, as well as other food substrates, by LAB has been shown to improve protein digestibility (Holzapfel, 1997; Taylor and Taylor, 2002), increase nutritional bioavailability of minerals and other micronutrients (Agarry et al., 2010; Grefferuille et al., 2011), prolong shelf life (Angelov et al., 2006; Gupta et al., 2010a), and finally enhance organoleptic qualities (Nionelli et al., 2014; Peyer et al., 2015).

The favourable macro- and micronutrients profile present in cereals have made them an excellent candidate for LAB fermentation (Blandino et al., 2003), providing the necessary carbohydrates, amino acids, peptides, nucleotides, vitamins, minerals, and fatty acids for their growth (Endo and Dicks, 2014). The bioavailability of nutrients that are usually bound as reserve molecules in the form of starch and proteins can be enhanced with the addition of malted cereals, either directly or by adding to the pool of hydrolytic enzymes with e.g. amylases, glucanases and peptidases (Gupta et al., 2010b;

Nionelli et al., 2014). Charalampopoulos et al. (2002) reported that the significantly higher levels of fermentable sugars in the form of glucose, fructose, maltose and sucrose (ca. 15 g/L) and free amino nitrogen (80 mg/L) of a liquid barley malt medium were among the main reasons for the better growth of the LAB on this medium compared to raw barley and raw wheat media (ca. 3-4 g/L and 15–27 mg/L, respectively). Similarly, Herrera-Ponce et al. (2014) documented that *Lactobacillus* strains could adapt faster and entered the exponential growth phase sooner when inoculated in germinated or malted oats media compared to simple raw oats.

Along with the traditional milk-based formulations, cereals and other food substrates (fruits, teas and vegetables) have been increasingly considered as ingredients for *functional beverages* that satisfy dietary lifestyles such as *veganism* and *allergen-free* (Corbo et al., 2014). In this regard, oats have received particular research interest because of their high content in soluble fibres e.g. *β*-glucan, linked to health-promoting effects on diabetes and cardiovascular diseases (Angelov et al., 2006; Herrera-Ponce et al., 2014). Oats formed the main substrate for the first commercialised cereal-based *probiotic* beverages, Proviva® (Skane Dairy, Sweden) and Yosa® (Bioferme, Finland), which both do not contain any milk constituents (Salovaara, 1996). On a worldwide basis, maize, rice and wheat are the prevailing crops in terms of area reserved for cereal cultivation and total cereal production (Poutanen, 2012). However, ancient and/or minor cereals, such as kamut, spelt, einkorn, millet, and sorghum, and pseudocereals, such as quinoa, amaranth, and buckwheat, have generated renewed interest, particularly in Western countries, because of their higher content in beneficial minor components (dietary fibre, resistant starch, minerals, vitamins, phenolic compounds) (Coda et al., 2014) compared to staple grains (wheat, maize, rice), and the possibility to fulfil further dietary needs, such as low-gluten or gluten-free (Zannini et al., 2012).

2.5.2 LAB fermentation for sensory improvement in cereal-based beverages

Raw cereals carry very low levels of organoleptic-active compounds, and in this form, give flat, "green" and unpleasant odours and flavours (Zhou et al., 1999). The bitterness and astringency carried by certain phenolic compounds found in the outer layers of whole grains can also lead to poor acceptance (Heiniö et al., 2011). Together with other preparation steps such as boiling, toasting, and roasting (Coda et al., 2011), fermentation has been used to improve sensorial and textural properties of liquid cereal substrates.

The fermentation of an oat-based substrate with a *Lb. plantarum* strain for the production of a yogurt-like functional beverage improved the sensorial characteristics by changing the earthy and raw notes of the untreated oat flakes into dairy notes and a pleasant sourness (Nionelli et al., 2014).

Table 3. Studies employing defined and characterised LAB as only starter cultures in controlled liquid cereal-based fermentations that focused on flavour and/or sensory-related attributes.

Topic of study	**Cereal substrate**	**Lactic Acid Bacteria**	**References**
Functional emmer beverage	Emmer flour, emmer gelatinised, emmer malt	*Lb. plantarum* 6E; *Lb. plantarum* 10E; *W. confusa*	(1)
Volatile profile and flavour stability	Barley malt	*Lactobacillus* spp.	(2)
Texture promotion and exopolysaccharides enrichment	Oat powder (Adavena® G40 and M40)	*Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772; *Lb. brevis* DSM 1269; *Lb. delbrueckii* subsp. *bulgaricus* DSM 20081; *S. thermophiles* DSM 20259	(3)
Oat-based, yogurt-like beverage	Oat powder (Adavena® G40)	*Pediococcus damnosus* 2.6; *Lb. delbrueckii* subsp. *bulgaricus*; *S. salivarius* subsp. *thermophilus*	(4)
Volatile analysis in *Bushera*	Sorghum	*W. confusa MINF8; Lb. paracasei MINF98; Lb. fermentum MINF99; Lb. brevis MINF226; Lb. plantarum MINF227*	(5)
Oat-based, yogurt-like beverage	Oat flakes flour (with enzymes addition)	*Lb. plantarum* LP01, LP06, LP09, LP32, LP39, LP40, LP48, LP51; *Lb. casei* LC10, LC11, LC03; *Lb. paracasei* LPC02, LPC16	(6)
Volatile profile and flavour stability	Barley malt	*Lb. brevis* R2Δ; *W. cibaria* PS2; *Lb. plantarum* FST1.7; *Lb. reuteri* R29	(7)

Volatile analysis from probiotic strain	Oat, wheat, barley, spray-dried malt extract	*Lb. plantarum* NCIMB 8826	(8)
Volatile analysis of probiotic formulations	Oat, barley, barley malt	*Lb. acidophilus* NCIMB 8821; *Lb. plantarum* NCIMB 8826; *Lb. reuteri* NCIMB 11951	(9)
Acceptance of probiotic cereal beverages	Starch-free extracts of flour–water suspensions prepared from oats and malt	*Lb. acidophilus* NCIMB 8821; *Lb. plantarum* NCIMB 8826; *Lb. reuteri* NCIMB 11951	(10)
Textural modulation in novel beverages	Barley malt	*W. cibaria* MG1	(11)

References in table: (1) Coda et al., 2011; (2) Krahl et al., 2009; (3) Mårtensson et al., 2002a; (4) Mårtensson et al., 2002b; (5) Muyanja et al., 2012; (6) Nionelli et al., 2014; (7) Peyer et al., 2015; (8) Salmerón et al., 2009; (9) Salmerón et al., 2014b; (10) Salmerón et al., 2015; (11) Zannini et al., 2013.

Investigations on flavour and sensorial changes due to LAB fermentation in liquid cereal substrates have been initially done in relation to off-flavour formation, unwanted acidification, hazes and sediments during microbial spoilage in beer (Bokulich and Bamforth, 2014). More recently, research has concentrated on the flavour and textural changes caused by the deliberate inoculation of LAB starter cultures as pure inoculum in cereal beverages (Table 3). These studies have been mainly carried out during the development of potentially *probiotic* cereal beverages (Coda et al., 2011; Salmerón et al., 2014a), or for quality improvement of traditional cereal-based fermented beverages (Blandino et al., 2003). Tropical fermented drinks are often affected by texture and flavour inconsistencies caused by the spontaneous, mixed microbiota found as inoculum during such fermentations (Nout, 2009). In an attempt to guarantee product safety and functionality of these products, without changing the sensorial characteristics, researchers have tried to replace the natural "*backslop*" cultures with defined cultures of single or mixed LAB. Agarry et al. (2010) changed the natural inoculum used for the production of *Kunun-zaki*, a spontaneously fermented non-alcoholic beverage traditionally produced in Nigeria, with a simpler starter culture comprising three

dominant LAB species found during natural fermentation, namely *Lb. plantarum*, *Lb. fermentum* and *Lact. lactis*. While the nutritional value could be increased by improving mineral bioavailability, the use of these defined starter cultures also led to a product with better appearance, aroma, taste and acceptability than the control sample. When the natural inoculum in *Gowé* beverage was replaced with individual LAB cultures of *Lb. fermentum* L025 and *W. confusa* L015, acidification of the substrate was obtained faster, helping the inhibition of growth of pathogens, while sensorial scores were comparable to the traditional beverage (Vieira-Dalodé, 2008). Nonetheless, similar attempts have also reported unsatisfactory results, obtaining products with a simpler or different sensorial profile than the original ones (Onyango et al., 2004). In this regard, the exclusion of yeast from the original starter culture has caused the loss of important flavour-active metabolites, especially higher alcohols and esters (Muyanja et al., 2012).

The majority of work done on novel liquid cereal-based fermented products has chosen *Lb. plantarum* as starter culture because it is robust under conditions of low pH (Charalampopoulos et al., 2002), which gives this strain a competitive advantage against other autochthonous microorganisms present on the grains. This species is also able to deliver a pleasant organoleptic profile in the form of "dairy"- and "fruity"- related flavours (e.g. diacetyl, acetoin, and acetaldehyde, respectively) (Prado et al., 2008; Salmerón et al., 2015). However, a defined strain does not preclude the release of specific flavours when inoculated in different cereal broths. The microbial flavour compounds released by *Lb. plantarum* NCIMB 8826 after fermentation of four different gruels (oat, wheat, barley and barley malt) were present at varying concentrations depending on the cereal used (Salmerón et al., 2009). Moreover, none of the metabolites were common for all substrates, indicating a complex flavour-formation interdependency that exists between bacterial culture and substrate components.

2.5.3 Flavour and taste formation by LAB

Carbohydrates, amino acids and other chemical compounds (e.g. organic acids, fatty acids) present in cereals, or released from LAB as intermediate compounds during fermentation, can be channelled into different metabolic pathways that ultimately lead to specific organoleptic-active compounds (Gänzle et al., 2007). As shown in Figure 3, the intermediate compound *pyruvate* is often the starting point for subsequent reactions, which end up releasing different flavour- and taste-active compounds (Liu, 2003). An

overview of the bacterial compounds found repeatedly during liquid cereal-based fermentations using LAB starter cultures as sole inoculum are listed in Table 4. The *non-volatile* fractions include primarily sugars and some carboxylic acids that contribute to the sweet and sour taste of the beverages. The *volatile fraction*, i.e. substances that tend to vaporise from the liquid bulk and are perceived as odour and flavour, comprises principally of other carboxylic acids, alcohols, aldehydes, ketones, and esters (Lasekan and Lasekan, 2012). Nevertheless, Table 4 does not consider the broad range of flavour-active compounds that are indirectly released from the substrate as a consequence of LAB fermentative activities (e.g. butyric acid, 2-butanol, benzaldehyde, phenyl acetate) (Blandino et al., 2003; Krahl et al., 2009; Salmerón et al., 2009).

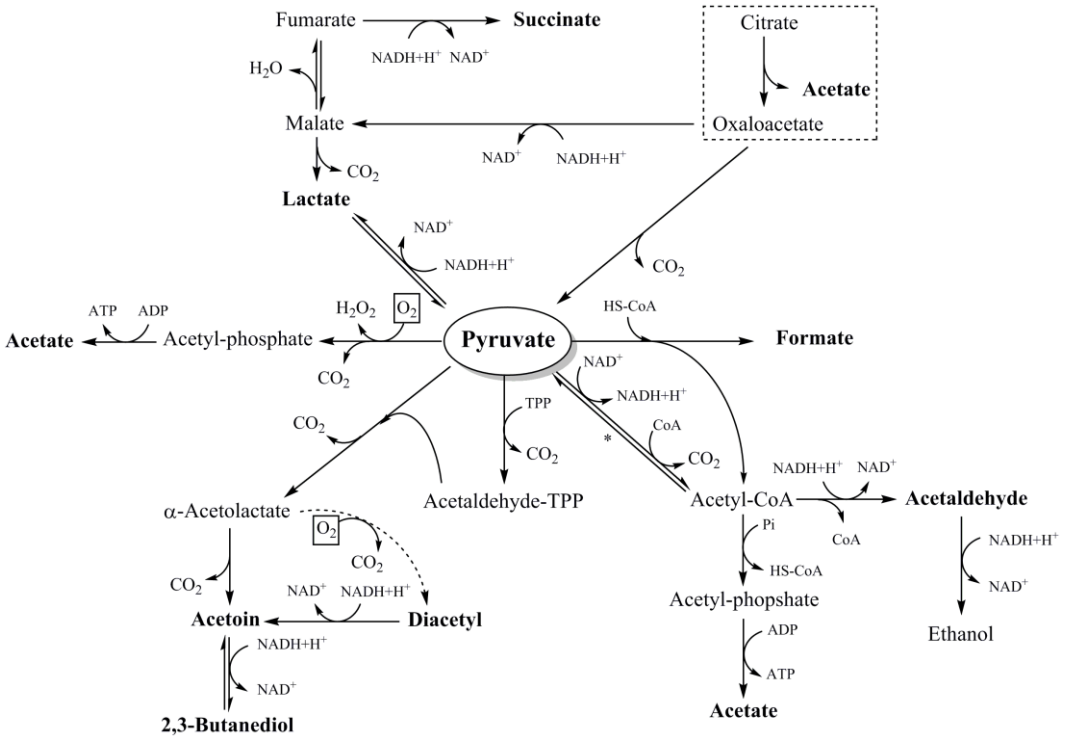

Figure 3. Overview of the main flavour-forming pathways from pyruvate found to play a major role in liquid cereal fermentation by LAB. Major flavour- and taste active end products are printed in bold. Utilisation of citrate is species-specific (dotted rectangle). The conversion from pyruvate to acetyl-CoA (*) can be inhibited by oxygen. Oxygen has been marked within a square in reactions relevant only under aerobic conditions. Diacetyl is formed by the non-enzymatic oxidative decarboxylation of α-acetolactate (dotted arrow). Adapted from Von Wright and Axelsson (2012), Gänzle (2015), and Hugenholtz et al., (2000).

Table 4. Overview of flavour and taste compounds repeatedly quantified in cereal-based beverages produced during controlled LAB fermentation as sole inoculum.

	Sensorial attributes*	Concentration range (fermentation time)	Aroma -/ Taste threshold*	References
Carboxylic acids				
Acetic	Cider vinegar, pungent	18 mg/L (4-5 h)- 650 mg/L (48 h)	15 mg/L (taste), 100 mg/L (aroma)	(1,2)
Lactic	Tart, acrid	180 mg/L (10 h)- 6600 mg/kg (96 h)	20 mg/L (taste)	(3,4)
Alcohols				
Ethanol	Alcohol, ethereal	0.51 mg/L (10 h)- 1600 mg/L (48 h)	0.008-0.9 mg/L (aroma)	(2,4,5)
Aldehydes / Ketones				
Acetaldehyde	Ethereal, green apples	0.10 mg/L (10 h)- 6.2 mg/L (36 h)	0.027 to 0.38 mg/L (aroma)	(4,5)
Acetoin	Yogurt, cream, butter	6.9 mg/L (48 h)- 115 mg/L (4-5 h)	50 mg/L (aroma)	(1,2)
Acetone	Solvent-like	0.2 mg/L (24 h)- 2.6 mg/L (36 h)	40 to 476 mg/L (aroma)	(4)
Diacetyl	Strong buttery, creamy	0.17 mg/L (48 h)- 0.38 mg/L (36 h)	0.005 mg/L (aroma), 50 mg/L (taste)	(2,4)
Esters				
Ethyl acetate	Fruity, sweet, brandy-like	0.04 mg/L (36 h)- 0.114 mg/L (24 h)	0.005 mg/L to 5 mg/L (aroma), 100 mg/L (taste)	(4)
Sugars				
Glucose	69 [a,**]	30 mg/L (48 h)- 57000 mg/kg (24 h) ***	11700 mg/L (taste) [a]	(6,7)
Fructose	114 [a,**]	50 mg/L (48 h)- ca. 6000 mg/L (18 h) ***	2400 mg/L (taste) [a]	(6,8)
Maltose	46 [a,**]	Traces (96 h)- 39000 mg/L (72 h) ***	13600 mg/L (taste) [a]	(3,8)

* Organoleptic description and thresholds of compounds in water were retrieved from Burdock (2002).

** Level is indicated as relative sweetness compared to sucrose (= 100) (10% aqueous solution) (Belitz et al., 2004).

*** Range for sugars is indicated as residual concentration after LAB fermentation.

[a] (Belitz et al., 2004).

References in tables: (1) Coda et al., 2011; (2) Peyer et al., 2015; (3) Muyanja et al., 2012; (4) Salmerón et al., 2014a; (5) Salmerón et al., 2015; (6) Charalampopoulos et al., 2002; (7) Mårtensson et al., 2002b; (8) Zannini et al., 2013.

For reviews on flavour and aroma compounds released by LAB in dairy and sourdough products with related metabolic pathways, see Smid and Kleerebezem (2014) and Salim-ur-Rehman et al (2006), respectively.

Non-volatile fraction

Since organic acids derive for the major part from the sugar metabolism by LAB, the increase in sourness coincides with a general decrease in sweetness (McFeeters, 2004), unless enzymatic release of sugar moieties from polysaccharides exceeds again the sugar consumption by LAB, leading the sweet taste to increase again (Mugula et al., 2003a). The "sour" perception of lactic acid in beverages carries important thirst-quenching properties and consequently has been exploited in novel refreshing products (Warner, 2010). Salmerón et al. (2015) added also to the importance of the final pH, often found between 3.0 and 4.5 after LAB fermentation, as a factor influencing the final acceptance of a novel beverage. In this case, higher pH values (> pH 3.55) positively correlated to higher acceptance of the beverages.

Lactic acid, quantitatively the most important organic acid found in LAB fermentation, is odourless but in aqueous solution imparts a mild acidic note (taste threshold of 20 mg/L in water), which is described as "tart" and "acrid" (Hartwig and McDaniel, 1995). Acetic acid, compared to lactic acid, is released in lower concentrations, but because of its lower taste threshold (15 mg/L in water) and higher volatility, it can become perceptible as pungent sour with a "cider-vinegar" aroma above a concentration of 100 mg/L (Burdock, 2002). The extent to which lactic and acetic acid accumulate depends primarily on the sugar metabolism of the starter culture and on the substrate supply, but fermentation conditions that can affect culture viability, e.g. temperature, buffering capacity (Helland et al., 2004), can determine the extent of acids released in the medium as well. Homofermentative LAB strains produce almost exclusively (> 90% theoretical yield) lactic acid from glucose (homolactic fermentation), while heterofermentative LAB release, besides lactic acid, also CO_2, acetic acid and/or ethanol (heterolactic fermentation) (Endo and Dicks, 2014). The major cereal disaccharides, i.e. maltose and sucrose, enter the cells through specific permease systems, where they then undergo

phosphorolysis, and enter both carbohydrate metabolic pathways as monosaccharides (Yun et al., 2003).

The metabolism of pyruvate into lactate is the dominant end reaction under anaerobic and aerobic conditions and when fermentable carbohydrates are abundant, as they are found mostly in cereal fermentations (Gänzle, 2015). However, if other compounds are present in the medium that can function as alternative electron acceptors, pyruvate can be channelled into alternative metabolic pathways (Liu, 2003). As an example, an increase in the oxygen input during LAB fermentation could be a steering point to increase the ratio of bacterial acetic to lactic acid released in the media. In this case, acetate is formed by the oxidation of pyruvate into acetylphosphate and concurrent synthesis of an additional ATP (Kandler, 1983). This pathway could be exploited in the production of novel fermented beverages such as "vinegar drinks" (Warner, 2010). The enrichment of the sweetening agent mannitol via reduction of fructose in heterofermentative LAB was the topic of many studies, as reviewed by Saha and Racine (2011). However, the rather low sweetness power of this compound compared to sucrose (50-52%), the associated laxative effects (Vrancken et al., 2010) and the simultaneous formation of acetate as co-product (Wisselink et al., 2002) do not speak for a valuable and efficient *in situ* enrichment in cereal-based beverages using wild-type LAB strains.

Volatile fraction

The main volatile compounds reported by studies on cereal-based liquid fermentation have been principally associated with the carbohydrate (mainly carboxylic acids and aldehydes, ketones, esters) and amino acid metabolism (mainly aldehydes and alcohols).

Diacetyl (butane-2,3-dione) is a vicinal diketone responsible for a butterscotch-like aroma and the very low odour threshold (0.005 mg/L in water) is often exceeded during LAB fermentation of cereal substrates (Burdock, 2002). This volatile is primarily formed from the oxidative decarboxylation of α-acetolactate (α-AL), an intermediate metabolite formed mainly during sugar, citrate and amino acid catabolism (Hugenholtz et al., 2000). Metabolically related to diacetyl are the less flavoursome acetoin, formed by the reduction of diacetyl or after enzymatic decarboxylation of α-AL, and 2,3-butanediol, which results from the reduction of acetoin (Axelsson, 1998). Although considered as being off-flavours in beer (Bokulich and Bamforth, 2013), these low-molecular weight

compounds are also responsible for mellowing the flavour during cereal fermentation (Mugula et al., 2003b). Only some LAB strains, e.g. *Lact. lactis, Lb. plantarum, Oenococcus oeni*, are able to metabolize citrate to pyruvate that can be eventually re-directed into the acetoin/diacetyl pathway (Hugenholtz, 1993). This pathway is responsible for the high accumulation of α-AL during LAB fermentation of milk-based matrices (citrate concentration of ca. 1500 mg/L) (Axelsson, 1998). Although citrate in cereal malt gruels has been reported as being rather low (170 mg/L) (Mandl, 1974), higher levels (871 mg/kg) have been reported in a sorghum-based liquid substrate (Muyanja et al., 2012). The complete depletion of the compound within 24 h of LAB fermentation was linked to an increase in acetoin and diacetyl production. Even higher levels of diacetyl and acetoin were found after LAB fermentation of a semi-liquid sorghum-based substrate (Mukisa et al., 2012). Because the medium contained no citrate, the accumulation of the two flavour compounds was attributed to an increased pyruvate metabolism by the LAB strains, caused by the good amylolytic activities of the co-culture. Finally, Salmerón et al. (2014a) found that diacetyl was only released in a malt-based medium upon fermentation by LAB starter cultures, but not in oats and barley media. However, it is not clear in this case if the higher amount of nutrients or other physico-chemical conditions were responsible for the accumulation of the aldehyde.

Acetaldehyde is a highly volatile aldehyde formed from pyruvate or threonine catabolism (Ardö, 2006). It has been described as delivering a pungent, fruity (green apples) flavour with sweet notes (odour threshold of 0.027 mg/L in water) (Mahattanatawee et al., 2005). Salmerón et al. (2015) showed that the high level of acetaldehyde (1.36 mg/L) found after fermentation of a malt-based beverage with *Lb. plantarum* NCIMB 8826 positively contributed to the high acceptance of the beverage. Muyanja et al. (2012) reported that the significant accumulation of acetaldehyde (2.54-4.45 mg/kg) using single LAB starter cultures during the fermentation of *Bushera* positively contributed to sweet notes in the early stages of fermentation.

Since ethanol has a neutral odour, it does not contribute directly to the overall flavour as a standalone compound (Berger, 2007). However, the presence of ethanol can influence the retention of other flavour compounds in solution, as it has been found in beer matrices for 3-methylthiopropionaldehyde, considered an off-flavour with a malty character and better retained at higher ethanol concentrations (Perpète and Collins, 2000). The level of ethanol found after LAB fermentation in cereals rarely reaches the

limit (0.5% (v/v) as widely acknowledged) required for non-alcoholic claims in fermented beverages (Kreisz et al., 2008). Values higher than this are normally attributed to the presence of wild yeasts in the fermentation (Muyanja et al., 2012). This aspect has to be taken into consideration when working with flours that were not previously heat-treated.

Amino acids play a central role as flavour-forming substrates for LAB (Gänzle et al., 2007). Besides possessing taste properties of their own (e.g. sweet, bitter, sulphurous and umami) (Solms, 1969), amino acids serve as substrate for Maillard reactions that can accumulate organoleptic-active carbonyl compounds, heterocycles as well as melanoidins (Pozo-Bayón et al., 2006). The by-products of amino acid catabolism in LAB have been repeatedly reported as important flavour-active compounds in liquid cereal-based fermentations (Coda et al., 2011; Mugula et al., 2003a). Muyanja et al. (2012) described a decrease in "malty" aldehydes (2-methyl-1-propanal, 2-methyl-1-butanal, 3-methyl-1-butanal) and an increase in the corresponding alcohols (2-methyl-1-propanol, 2-methyl-1-butanol, and 3-methyl-1-butanol) with fruity and alcoholic flavours upon fermentation of *Bushera*. The same compounds were considered to provide similar flavour aspects in *Togwa*, a fermented beverage prepared either from cassava, maize, sorghum, millet or their combinations (Mugula et al., 2003b). The above mentioned aldehydes and alcohols can be released from the catabolism of the branched amino acids leucine, isoleucine, and valine after conversion into the respective α-keto-acids by means of aminotransferases, and subsequent decarboxylation into aldehydes (Ardö, 2006). The reduction of these compounds into alcohols, however, has been often assigned to indigenous yeasts present in the raw cereals (Muyanja et al., 2012). On-going microbial acidification of an untreated cereal substrate can lead to better activation of the endogenous proteinases and peptidases (pH optimum between 4 and 5 in wheat, rye and barley) (Belitz et al., 2009), while other LAB can actively increase the fermentable nitrogen level providing exogenous proteases (Coda et al., 2012; Thiele et al., 2002). The latter have been reported to be rather low compared to LAB strains typically involved in dairy fermentation, as it was reported for LAB strains associated with *Boza*, a viscous drink based on wheat, millet or maize commonly found in Eastern Europe and Turkey (Kivanc et al., 2011). Higher proteolytic activites (i.e. aminopeptidase and proteinase) during the fermentation of *Togwa* have been attributed to the natural inoculum, composed of a mixture of both microbial and yeast cultures, rather than to single LAB starter cultures (48). A similar co-fermentation condition between yeast and LAB have

been found to increase protein digestibility in *Ogi*, a fermented maize-based beverage (53). To counteract the sometimes low proteolytic activity of cereal-associated LAB strains in these beverages, malt has been supplemented to the gruels to directly lead to more free amino acids and peptides available for the starter cultures (Mugula et al., 2003c).

Finally, free fatty acids such as oleic and linoleic acid can act as precursors for potent flavour compounds such as methylketones, alcohols, and lactones (Smit et al., 2005). However, because of the generally low lipolytic activities of LAB cultures, these volatiles are formed by other microorganisms associated with food preparation, e.g. moulds in cheese. Because of the relatively low concentration of lipids in cereals, volatiles derived from lipolysis metabolism have not been studied in detail during liquid cereal fermentations.

Flavour stability

Few recent studies have been conducted to assess how the flavour of LAB-fermented cereal beverages might change over time because of the accumulation of staling compounds or the degradation of positive organoleptic attributes. Krahl et al. (2009) tested the flavour stability of a commercial and an experimental barley malt-based beverage (7% (w/w) extract) fermented with a *Lactobacillus* spp. by applying a forced-ageing treatment that combined shaking and warm storage in order to simulate a 3-4 months natural ageing. The authors found that ageing could be described by the concentration changes of eight indicator substances (3-methylbutanal, 2-furfural, benzaldehyde, 2-phenylacetaldehyde, hexadienal, heptanal, methional, and β-damascenone) related to thermal and/or oxidative stress. Moreover, they noted that inclusion of fruit concentrates and aroma compounds contributed to the amounts of stale flavour compounds. In a similar study, Peyer et al. (2015) found that the sensory characteristics of fermented malt-based worts (6% (w/w)) (*Lb. plantarum* or *Lb. reuteri*) showed higher stability to ageing compared to an unfermented control. However, a significant increase of ageing-related flavour substances such as 2-furfural ("almond"), followed by the Strecker-aldehydes 2-phenylacetaldehyde ("green"), 2- and 3-methylbutanal (both "malty") and γ-nonalactone ("coconut") (Berger, 2007), could not be fully prevented in any of the samples.

2.5.4 Texture modulation

Exopolysaccharides and improved mouthfeel

Some LAB are able to excrete high molecular weight polysaccharides that can increase the viscosity of the liquid substrate. Exopolysaccharides (EPS) are formed through polymerisation of sugar subunits and can be either composed from repeating glucose or fructose subunits (= homopolysaccharides) or from two or more different subunits (= heteropolysaccharides) (Galle and Arendt, 2014). The biochemical reactions involved in the formation of both exopolysaccharides has been extensively reported for sourdough LAB in the review of Tieking and Gänzle (2005).

Their contribution through *in situ* production of EPS is of particular interest to manufacturers of fermented cereal-based drinks aimed to imitate dairy products (Bernat et al., 2014). These products can therefore make a "natural", "additive free" claim and, at the same time, avoid the costs that result from the expensive and laborious EPS purification procedures (Badel et al., 2011). Coda et al. (2011) managed to increase the textural properties of a beverage formulated with gelatinised emmer flour (30% (w/w) in tap water) and added sucrose (10% (w/w)) using EPS-forming species of *W. cibaria* as inoculum. The 4-fold viscosity increase, compared to a control fermented by EPS-negative *Lb. plantarum* strain, conveyed a texture similar to yogurt to the final product. Similarly, Mårtensson (2002b) studied the possibility of developing a yoghurt-like ropy product derived entirely from oats and water by employing an EPS-producing *Pediococcus damnosus* strain in combination with an ordinary yoghurt starter culture. A sensory preference test successfully showed no significant difference between the flavoured, non-dairy product and a dairy equivalent control. The yield of exopolysaccharides can be regulated by changing the concentration of sugars in the medium. A study by Zannini et al. (2012) examined the potential of two cereal-associated *W. cibaria* strains to produce exopolysaccharides *in situ* during the development of a prebiotic drink based on barley malt extract. The authors found that the strain *W. cibaria* MG1 was able to accumulate ten times more dextran (up to 14.4 g/L) when the substrate was supplemented with 10% sucrose compared to unsupplemented wort (1.4 g/L). The higher viscosity positively influenced the mouthfeel of the beverage. Dextran, a flavourless homopolysaccharide composed of glucose subunits, is a GRAS-granted thickener already used by the food industry. The ability of LAB to release texture-enhancing EPS has been found in many starter cultures involved in the production of traditional

beverages. Adebayo-tayo and Onilude (2008) reported that a significant number of LAB strains involved in the fermentation of traditional Nigerian *Ogi* (sorghum-based) and *Fufu* (cassava-based) were EPS producers. Out of 264 isolates of *Lb. fermentum* involved in the spontaneous fermentation of two West African sorghum beers *Dolo* and *Pito,* up to 89% had the ability to produce EPS (Sawadogo-Lingani et al., 2008). The thicker texture of *Dolo* was considered an important product quality for its sensorial appeal.

The type of EPS produced and its amount depends principally on the sugars present in the medium (Galle and Arendt, 2014), which can act as substrate or as acceptor molecules, on the presence of micronutrients (e.g. minerals acting as enzymes co-factors), and the environmental conditions (e.g. incubation temperature and time). *Lb. delbrueckii subsp. bulgaricus* NCFB 2772 was found to enhance to a greater extent the viscosity of an oat-based medium when glucose was present as a supplementary carbon source instead of fructose, which was seen to cause the release of EPS with lower relative molecular mass (Grobben et al., 1997). Additionally, the authors showed that the combination of longer incubation time and lower temperature, together with a favourable carbon/nitrogen ratio, were beneficial for a substantial EPS production. The amount of EPS also can prevent physical instability and phase separation of the final beverage (Mårtensson et al., 2002b). It should be noted, however, that an increase in the viscosity has been reported to eventually decrease diffusion and release rate of aroma and flavour compounds within the matrix (Saint-Eve et al., 2006).

Amylases and reduced viscosity

Some cereal flours, when mixed with water, can lead to undesirable thick, porridge-like textures, because of the high content of molecules like starch and β-glucans that have a viscosity-enhancing effect (Lorri and Svanberg, 2009). This is especially important in certain African countries, where maize, sorghum, or millet porridges represent a crucial energy source as weaning food for young children (Humblot et al., 2014). In order to maintain a high-energy density in these formulations without the need for watering down, LAB cultures with enzymatic activity have been employed for partially degrading these biopolymers (Onyango et al., 2004). Among these, *amylolytic* LAB (ALAB) able to degrade polysaccharides have been isolated from many traditional beverages (Guyot, 2012). The biodiversity of ALAB is quite limited and the most prominent belong to the species *Lb. manihotivorans, Lb. fermentum, Lb. amylovorus, Lb. amylophilus, Lb. plantarum and Lb. amylolyticus* (Reddy et al., 2008). A fundamental role was seen to be played by the

gene *amyA*, which encodes for an extracellular α-amylase and is shared among ALABs. In a recent study, Humblot et al. (2014) measured the gene expression and enzymatic activity of a number of starch-degrading enzymes (α-amylase, α-glucosidase, neopullulanase, amylopectin phosphorylase, and maltose phosphorylase) produced by *Lb. plantarum* A6 during fermentation of a pearl millet-based gruel. The highest expression levels for *amyA* correlated with the strongest liquefaction effect. The same strain was previously used in combination with an indigenous inoculum for the production of *Ben-saalga*, a popular traditional fermented gruel based on a pre-cooked pearl millet and groundnut slurry (Songré-Ouattara et al., 2009). The authors obtained a high-energy density beverage with the desired liquid consistency replacing malt as liquefaction aid. Although cereal malts have been used as a cheap and quick way to reduce the viscosity of such beverages (Tou et al., 2007), the variability in their amylolytic activity and the time needed for their production (several days) have been identified as some of their drawbacks (Songré-Ouattara et al., 2009). Because it was found that high levels of glucose, maltose, or sucrose can inhibit the synthesis of amylases by ALAB, Mukisa et al. (2012) applied a co-culture comprised of a strong ALAB with a fast-fermenting LAB in order to accelerate the depletion of free fermentable sugars. The combined culture was faster in hydrolysing starch and reducing viscosity compared to a strong ALAB monoculture or to malt addition. At the same time, the fast acidification and high lactic acid yields could ensure better safety and stability of the products.

2.5.5 Future trends

Metagenomic analyses have allowed us to increase our understanding of the complex ecosystem that is present in many traditional microbial fermentations, and have served to highlight the complex interactions involved in the formation of flavour and aroma compounds. With "*omic*" technologies becoming more affordable, an increasing number of studies are approaching flavour and textural improvement of starter cultures through regulation and manipulation of their metabolic systems (Papagianni, 2012). The metabolism of pyruvate is a crucial steering point in the microbial production of flavours, and therefore has been often subject to metabolic engineering intervention. Common strategies have included the re-routing of metabolic pathways by enhancing and/or impairing enzymatic reactions, e.g. for the overproduction of mannitol (Wisselink et al., 2002) or by direct cloning of genes involved in specific flavour

compound production (Chaves et al., 2002; Nadal et al., 2009). In this regard, LAB are considered as an excellent candidate for metabolic engineering strategies aimed to overproduce compounds of interest, because they possess relative simple energy and carbon metabolisms, and a small genome size compared to other microorganisms like yeast or fungi (Papagianni, 2012). The dairy industry has spent many years of significant effort in tailoring starter cultures for defined flavour and textural benefits. Especially *Lact. lactis* strains have been widely used as model organisms (Kleerebezem and Hugenholtz, 2003). In the effort of enhancing the production of flavour compounds associated with "buttery" notes, such as diacetyl, Hugenholtz et al. (2000) engineered a *Lact. lactis* strain impaired in its ability to reduce α-AL into acetoin. Under favourable oxidative conditions, the metabolic flux was rerouted towards direct decarboxylation of α-AL into diacetyl, which could be accumulated up to 5 times under aerobic compared to anaerobic conditions. In an attempt to naturally enrich the sweetness of a lactose-based substrate, the glucose metabolism of *Lact. lactis* spp. *cremoris* was disrupted by deletion of the responsible genes. This led to the extracellular accumulation of glucose, and a final increased sweetness (Pool et al., 2006).

On the other side, metabolic engineering strategies used for controlling the amount and type of EPS produced have focused on the over-production of their precursors, i.e. sugar nucleotides (Kleerebezem and Hugenholtz, 2003), or the insertion of different glycosyltransferases-encoding genes (Kleerebezem et al., 1999). Li et al. (2015) managed to increase EPS production in *Lb. casei* LC2W by 46% compared to the wild-type strain through overexpression of NADH oxidase, suggesting that more carbon source was directed towards EPS production rather than used for growth and lactate generation.

While such genetic modification procedures can, in principle, be used to increase certain flavour, aroma or textural compounds, the method of strain transformation is important when considering final application in food products.

2.6 Concluding remarks

Maltsters and brewers worldwide could avail of LAB as efficient cell factories for the production of functional ingredients to enhance the quality of malting cereals and related beverages (Waters et al., 2013). Depending on the desired application, suitable LAB starter cultures could be added to wort to enrich a wide range of antimicrobials/antifungals compounds, high amounts of lactic acid, or attractive organoleptic compounds. In addition, by-products resulting from the malting and brewing operations could be re-valorised through fermentation, decreasing manufacturing costs while improving sustainability goals.

LAB fermented worts play an important role as biopreservants with potential to replace synthetic broths and for direct application during malting and other food processing. The LAB-fungal interaction is complex in nature and the final inhibition is a combined action of different hurdles of biochemical and physical characters (Schnürer and Magnusson, 2005). Although nutrient addition and external buffer / neutralising agents can be added during lactic acid hyperproduction in wort, these should be kept to a minimum in order to improve cost-efficiency of the process. In this regard, the screening of the starter culture should be prioritised for desired metabolic abilities such as amylolytic activity, low pH resistance, limited auxotrophy and lack of by-products.

The development of functional fermented cereal products through application of defined LAB meets the current demand for healthier and diversified foods. The full prediction of the final sensory attributes, however, is still a very complex task due to dynamic interactions between starter cultures, substrate and fermentation conditions. If these studies are combined with flavour and sensory analysis, the results could deliver important information on the functionality and fitness of a starter culture in a particular cereal-based fermentation, with the ambitious aim to predict specific flavour and textural profile formation.

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Chapter 3: Inhibition of *Fusarium culmorum* by carboxylic acids released from lactic acid bacteria in a barley malt substrate

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3.1 Abstract

The effect of carboxylic acids, composed of both organic and phenolic acids, released in a barley malt substrate fermented by lactic acid bacteria was tested against *Fusarium culmorum* macroconidia and compared under different fermentation conditions. Phenolic acids released by *Lactobacillus plantarum* FST1.7 and *Lb. brevis* R2Δ were quantified using a QuEChERS method coupled with a HPLC-UV/PDA system. Their concentration improved with increasing extract content of the barley malt-based substrate and reached maximal concentrations after 48 h of fermentation performed at optimum growth temperature. Generally, phenolic acids were produced at levels far below their minimal inhibitory concentration (MIC), and limited synergistic effects were observed when mixed with organic acids. The fungal growth suppression by the wort fermented by *Lb. brevis* R2Δ (95 ± 9 h total inhibition) could be fully explained by the presence of antifungal carboxylic acids, whereas only partially accounted for *Lb. plantarum* FST1.7 (198 ± 19 h). Organic acids were mainly responsible for the ability of LAB fermented wort to cause fungal inhibition, whereas phenolic acids took only a secondary role at the low concentrations released. Longer fermentation times favoured primarily organic acid release, whereas fermentation of higher malt extract substrates encouraged both organic and phenolic acids production. The understanding on how synergy works between antifungal compounds could help to identify strategies to further increase their concentration in wort, with potential to replace synthetic broths for direct application during food manufacturing.

3.2 Introduction

Competition between populations of microorganisms for limited resources available in a common habitat has led to the development of species-specific mechanisms targeted at inhibiting competitors (Hibbing et al., 2010). Due to their important history of application as starter cultures, especially in the food industry, lactic acid bacteria (LAB) have been intensively studied as bioprotective agents. They produce a wide spectrum of antimicrobial compounds in form of e.g. organic acids (e.g. lactic acid, acetic acid, propionic acid) (Dang et al., 2009), low molecular weight compounds (e.g. diacetyl) (Aunsbjerg et al., 2015), proteinaceous metabolites (e.g. cyclic dipeptides) (Magnusson and Schnürer, 2001; Niku-Paavola et al., 1999; Ström et al., 2002), bacteriocins (e.g. nisin, reuterin) (Reis et al., 2012), and hydroxy fatty acids (Black et al., 2013). Among these, the family of phenolic acids has gained interest (Axel et al., 2014; Brosnan et al., 2012; Oliveira et al., 2014; Svanström et al., 2013), as several of these low molecular weight compounds (e.g. 3-phenyllactic acid and benzoic acid) have been shown to retard or eliminate fungal growth both *in vitro* and *in situ*. The overall antimicrobial activity of LAB has been attributed to synergistic interactions between the aforementioned compounds rather than to single compounds (Axel et al., 2016; Crowley et al., 2013), making a complete understanding of the final effect complex (Dalié et al., 2010).

Numerous studies have previously reported the ability of strains of *Lb. brevis* and *Lb. plantarum* to successfully inhibit fungal growth in cereal substrates (Laitila et al., 2002; Lavermicocca et al., 2000). While strains of the *Lb. brevis* were found to exhibit broad antifungal activity against *Fusarium* species (Mauch et al., 2010), *Lb. plantarum* FST1.7 was previously employed to increase the shelf life of sourdough bread by inhibiting the outgrowth of *Fusarium* spp. in wheat bread (Dal Bello et al., 2007) and gluten-free bread (Moore et al., 2007). *Fusarium culmorum* is one of the most detected fungal isolates in cereals (Parry er al., 1995) and responsible for under-development and necrosis in small grains (e.g. Fusarium Head Blight) (Champeil et al., 2004; Wagacha and Muthomi, 2007). This species is able to release mycotoxins (e.g. deoxynivalenol, nivalenol, zearalenone) and to cause technological failures in food production (e.g. gushing in beer and decreased yields during malting) (Nierop and Rautenbach, 2006; Stübner et al., 2010). For these reasons, *F. culmorum* was repeatedly chosen as a fungal target in studies aimed in increasing safety and shelf-life of cereal-based products (Axel et al., 2016; Dal Bello et al., 2007; Oliveira et al., 2014; Ryan et al., 2011).

The type and amount of antimicrobial compounds released by LAB have been described as strain-dependent (De Muynck et al., 2004; Valerio et al., 2004). However, other conditions, e.g. substrate type, presence of co-factors, fitness of the culture, temperature, etc. can play a role (Dalié et al., 2010; Laref et al., 2013). The majority of studies aimed at quantifying antifungal compounds released by LAB have been performed using synthetic growth media, e.g. deMan Rogosa and Sharp (MRS). The direct application of these media into food matrices is limited as many of these formulations include ingredients unfit for human consumption and/or are of animal origin. The use of unconventional raw materials to enrich fungal inhibitors is attractive because of the clean-label nature (Pawlowska et al., 2012; Pitt and Hocking, 2009). In this regard, barley malt wort is a nutrient-rich substrate with multiple uses such as for flavouring, colouring, sweetening agents in food manufacturing (Bender, 2006).

This study aims to provide further evidence on the role of carboxylic acids, with a main focus on phenolic acids, on the overall antifungal activity by LAB, and to unravel part of the complex synergistic interactions occurring with organic acids. To this end, fungal inhibition by active metabolites released during LAB fermentation of a malt-based substrate was determined. The level of fungal inhibition, expressed as minimal inhibitory concentration (MIC), by the single compounds and mixtures of these were assessed against the same target organism. In addition, the impact of different fermentation conditions on the accumulation of carboxylic acids was also investigated.

3.3 Materials and methods

3.3.1 Bacterial and fungal cultures

The antifungal-positive strains *Lactobacillus plantarum* FST1.7 (Dal Bello et al., 2007; Moore et al., 2007) and *Lb. brevis* R2Δ (Axel et al., 2014), the antifungal-negative control strain *Lb. brevis* L1105 and the fungal species *Fusarium culmorum* FST 4.05 were obtained from the UCC culture collection (School of Food and Nutritional Sciences, University College Cork, Cork, Ireland). They were kept as frozen stocks in 80% glycerol at -80°C. The bacterial cultures were routinely cultivated on MRS agar plates (Merck, Darmstadt, Germany) under anaerobic conditions for 48 h at 30°C. The 16S rRNA gene of the *Lb. plantarum* FST1.7, *Lb. brevis* R2Δ and L1105 strains showed 99% identity to the 16S rRNA gene of *Lb. plantarum* DSM 13273^{T} and *Lb. brevis* DSM20054^{T} type strains, respectively. Cultivation of the fungus and collection of the spores were done according to Mauch et al. (2010). All reagents used were analytical-grade from Sigma-Aldrich, Missouri, USA, unless otherwise stated.

3.3.2 Barley malt wort production

Commercial malt made from spring barley (*Horderum vulgare*, variety Propino, harvest 2012) (Malting Company of Ireland Ltd., Cork, Ireland) was ground using a two-roller mill (0.8 mm distance gap) to obtain 8.8 kg of grist for barley malt extract (wort) production. The grist-to-water ratio was set at 1:3.52, and the following mashing regime was performed using a pilot-scale (60 L) brewhouse: 20 min at 50°C, 40 min at 62°C, 20 min at 72°C, and 5 min at 78°C for mashing-off. After removing the spent grains from the wort during lautering with two sparging steps (10 L each), the wort was boiled for 30 min and adjusted to a final extract content of 12% (w/w) before being filled into 19 L stainless steel containers and kept for short-term storage at 0°C.

3.3.3 Fermentation of wort-based substrate

Tempered wort (12% (w/w)) was adjusted to an extract content of 9% (w/w), 6% and 3% by dilution with sterile water. The LAB strains were propagated twice in 10 ml MRS broth (24 h at 30°C), centrifuged (2000 x g for 10 min) and washed with Ringer's solution before inoculation in wort and modified MRS (mMRS) (8.0 log cfu/mL for 48

h at 30°C). mMRS medium was formulated as MRS (43) without sodium acetate (CH_3COONa) and potassium dihydrogen phosphate (KH_2PO_4). Fermentation trials were done in triplicate using sterile 50 mL screw-cap tubes under static conditions for a total duration of 120 h. Long fermentation times were chosen in order to follow post-stationary phase acidification and inhibition provoked by cell lysis. Bacterial growth in diluted worts and mMRS was measured every 24 h by serial dilution and plating over the total duration of fermentation duration (120 h). Simultaneously, acidification kinetics were analysed by measuring total titratable acidity (TTA) (0.25 M NaOH titration until pH 8.1), expressed as concentration of hydrogen ions (mmol/L), and pH. Cell-free supernatants (cfs) of the fermented substrates were obtained after centrifugation (2000 x g for 10 min) and subsequent sterile filtration (0.45 µm MINISART®-plus filter, Sartorius Stedim Biotech GmbH, Goettingen, Germany). While a part of the cfs was used for the challenge tests *in vitro,* the rest was stored at -20°C for HPLC analysis.

3.3.4 Organic acids, sugars and ethanol content

An Agilent 1260 HPLC system equipped with an ultra violet diode array detector (UV/DAD) and a refractive index detector (RID) was employed to analyse organic acids, sugars and ethanol. External standards for organic acids (lactic, acetic and propionic acid), sugars (maltotriose, maltose, glucose, fructose) and ethanol were used for calibration curves (linear correlation coefficient $R^2 > 0.99$). Fermented samples were clarified with 7% perchloric acid overnight (16 h, 4°C), centrifuged (9000 x g, 5 min), and sterile-filtered through a 0.20 µm pore size filter (CHROMAFIL® Xtra, Macherey–Nagel, Germany). The measurements were performed using an Agilent Hi-Plex H (7.7 × 300 mm, pack size 8 µm, Agilent, Cork, Ireland) column equipped with a PL Hi-Plex Guard column (7.7 × 50 mm, 8 µm, Agilent, Cork, Ireland). Twenty µL of the filtrates were injected and eluted isocratically with a flow rate of 0.5 mL/min using 0.05 M H_2SO_4 as mobile phase. The column was kept at 65°C and the samples were detected during a period of 30 min by a diode array detector set at 210 nm for organic acids and a refractive index detector for sugars and ethanol. Each fermentation condition was injected in triplicates.

3.3.5 HPLC of phenolic acids

The following 15 phenolic compounds were analysed: catechol, hydroxyphenyllactic acid, 4-hydroxybenzoic acid, hydrocaffeic acid, caffeic acid, phloretic acid, hydroferulic acid, *p*-coumaric acid, ferulic acid, benzoic acid, salicylic acid, hydrocinnamic acid, methylcinnamic acid (Sigma-Aldrich, Missouri, USA), vanillic acid (Fluka Chemie AG, Buchs, Switzerland), and 3-phenyllactic acid (Bachem Ltd., Merseyside, UK). Sample preparation was done according to the method described by Brosnan et al. (2014), with minor modifications. Briefly, the fermented samples were centrifuged for 10 min at 9300 x g and 10 mL of the supernatant were sterile-filtered (0.45 μm filter) and added to a tube together with 1 g NaCl, 4 g $MgSO_4$ and 10 mL ethyl acetate (containing 1% formic acid). The mixture was immediately hand-shaken for 1 min and centrifuged for 10 min at 400 x g. A QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) cleanup, usually done for multi-residue analysis of pesticides, drugs, and toxins (60), was applied to the upper organic phase. The mixture was vigorously shaken for 1 min, followed by another centrifugation step (10 min at 400 x g). A total of 5 mL of the organic solvent supernatant was then transferred in a glass tube containing 100 μL dimethyl sulfoxide (DMSO) and dried in a vacuum centrifuge (Scanspeed 32 and Vacsafe 15, Scanvac, Lynge, Denmark) for 2 h at 30 x g and 45°C. The volume left in the glass tube was reconstituted with H_2O/ACN (90/10, 400 μL) and sterile-filtered (0.20 μm pore size filter) into a HPLC vial. A volume of 10 μL was injected through a Gemini C18 column (150 Phenomenex, Macclesfield, UK) maintained at 30°C. The eluent was kept at a constant flow rate of 0.2 mL/min in gradient mode and composed of solvent A (H_2O with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid) and run as follows: 0 min – 5% B, 15 min – 15% B, 35 min – 40% B, 45 min – 95% B, 50 min – 5% B, 70 min – 5% B). The compounds were analysed using the UV/DAD detector and quantification was done at a wavelength of 210 nm. The metabolites were identified by comparing their UV absorption spectra at the same wavelength. Recovery rates of the phenolic compounds were done with 3.0 mg/L of each analyte added in wort and in chemically acidified wort (pH 3 with 0.1 M HCl), and ranged from 89.1% (hydrocinnamic acid) to 118.1% (vanillic acid) of the total spiked amount.

3.3.6 Impact of LAB fermented media on growth of *Fusarium culmorum*

An overlay plate assay as described by Axel et al. (2016) was done on mMRS agar to confirm the *in vitro* antifungal nature of the strains. For the microtiter plate assay, aliquots of 150 µL filter-sterilised fermented wort and mMRS were filled in the wells of a sterile 96-well microplate (Sarstedt AG & Co, Nuembrecht, Germany) and challenged with 4 log spores/mL of *Fusarium culmorum*. The plates were sealed with optically clear foil (Sarstedt, Nümbrecht, Germany) to prevent evaporation and incubated at 25°C with a constant agitation (every 5 s for 1 s) inside a Multiskan FC microplate reader (Thermo Scientific, Waltham, USA). The optical density at 620 nm (OD_{620}) was recorded on an hourly basis to assess the fungal growth curve.

3.3.7 Minimal inhibitory concentration of antifungal compounds against *F. culmorum* spores

In order to study the pH-dependent nature of the carboxylic compounds released by LAB, minimal inhibitory concentrations (MIC) were assessed with and without correction to pH 3 (0.1 M HCl) in Malt Extract Broth (MEB) (Difco Laboratories, Detroit, MI, USA) formulated as follows: malt extract, 6.0 g/L; maltose, 1.8 g/L; D-glucose, 6.0 g/L; yeast extract, 1.2 g/L (pH set to 5.45). To completely dissolve the phenolic compounds, the solutions were heated to 50°C and exposed to ultrasonic waves (Ultrasonic cleaner, VWR International, Darmstadt, Germany) for 1 min while kept in the dark. A concentration range from 30 to 30000 mg/L was supplemented in MEB. For the purpose of this study, the MIC was defined as the lowest concentration of antifungal compound needed to achieve no increase in absorbance (i.e. 100% inhibition) after 3 days of incubation at 25°C when inoculated with 4 log spores/mL of *Fusarium culmorum*. MICs were evaluated from three independent experiments using the microtiter plate assay as described above.

3.3.8 Spiking experiments in wort and malt extract broth

The levels of phenolic compounds and organic acids released after fermentation of wort (6% (w/w), 30°C, 48 h) by *Lb. plantarum* FST1.7 and *Lb. brevis* R2Δ were reconstituted in unfermented wort and MEB to determine their cumulative contribution to the overall fungistatic effect. For this purpose, both media were also adjusted to the pH levels

found after fermentation using 0.1 M HCl or 0.1 M NaOH. The inhibition of 4 log spores/mL of *F. culmorum* was assessed spectrophotometrically, as explained above.

3.3.9 Statistical analyses

Biological triplicates ($n = 3$) were done for all fermentations. One-way ANOVA on Minitab software (Version 17.0) was used to compare means between concentrations from different fermentation treatments. When F-values were found significant, Tukey's multiple comparisons procedure was used to further determine any significant differences between them. The level of significance was determined at $P < 0.05$. Results are expressed as mean ± standard deviation.

3.4 Results

3.4.1 *In vitro* antifungal activity using agar plate overlay method

The inhibition zones ("halos") surrounding the LAB cultures showed varied inhibition of *F. culmorum* (Figure 4A-C). The strongest suppression of mould growth was observed with *Lb. brevis* R2Δ (a), while the antifungal-negative *Lb. brevis* L1105 (c) failed to inhibit mycelia growth. *Lb. plantarum* FST1.7 (b), in contrast, inhibited the mycelia to a lesser extent then *Lb. brevis* R2Δ. In this case, the mould surface showed a flatter and smoother appearance than seen for both *Lb. brevis* strains. This altered growth pattern spread over the whole mycelia hints to a better distribution of the antifungal compounds released by *Lb. plantarum* FST1.7 within the agar plates, ultimately retarding mould growth rather than inhibiting it.

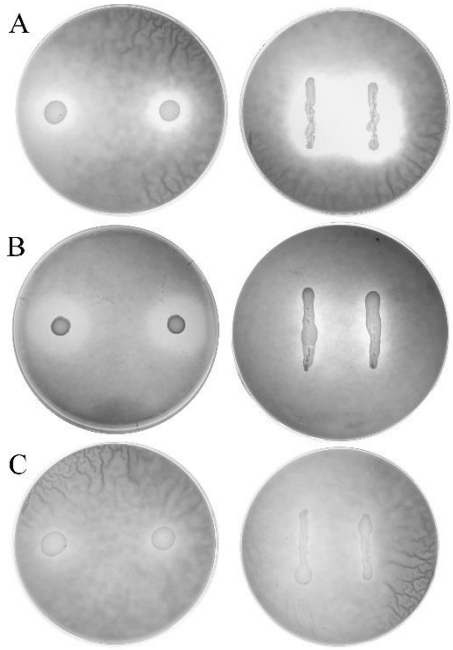


Figure 4. Zones of inhibition formed around the antifungal-positive *Lb. brevis* R2Δ (A) and *Lb. plantarum* FST1.7 (B) against *F. culmorum* spores (3 days of incubation) compared to the antifungal-negative control *Lb. brevis* L1105 (C).

3.4.2 Growth and acidification kinetics

As previously shown in other studies (Charalampopoulos et al., 2003; Oliveira et al., 2014; Salmerón et al., 2014), barley malt extract (wort) provides sufficient nutrients for substantial LAB growth. A high inoculum level of LAB starter culture (8 log cfu/mL) was chosen in order to ensure immediate dominance of the strain in the wort substrate. Compared to growth in mMRS, exponential growth was delayed by approximately 5-8 hours for the LAB strains grown in wort, and maximal cell densities were reached after 14–16 h and 18–21 h in mMRS and wort, respectively. *Lb. plantarum* FST1.7 adapted faster to the wort medium and grew to a higher extent than either *Lb. brevis* R2Δ and L1105, reaching a cell count of 10.7 log cfu/mL after 48 hours (compared to 9.48 and 9.38, respectively) in 6 % (w/w) wort (Table 5). A drop in viability was observed between 96 h and 120 h for both *Lb. brevis* strains in wort, with cell counts decreasing by 1.34 to 1.65 log cfu/mL, while *Lb. plantarum* FST1.7 maintained a constant viability until the end of the fermentation period. This could be attributed to the particular good acid resistance of this LAB species (Charalampopoulos et al., 2003). All strains were able to maintain high cell counts until the end of the fermentation period in mMRS, as previously shown by Oliveira et al. (2014) with *Lb. reuteri* and *Lb. amylovorus*. Acidification of the media followed the growth trend of the strains, and continued even after the strains entered the decline phase. Starting at a pH value of 5.77 ± 0.04 in wort, the pH dropped drastically within the first 24 h to a range between 3.97 (*Lb. brevis* L1105) and 3.27 (*Lb. plantarum* FST1.7) (31.2 to 43.6% decrease) (Table 5). Between 48 and 120 h of fermentation, the pH displayed a lesser, but constant decline (3.2-4.6% decrease), and settled to final pH values of 3.53-3.12. Total titratable acidity significantly ($P < 0.05$) increased within the first 24 h of LAB fermentation in wort, and stopped for *Lb. brevis* R2Δ and L1105 after 96 h of fermentation (48.0 ± 3.6 and 36.7 ± 2.3 mmol/L H^+ at 120 h, respectively), while it continued to increase for *Lb. plantarum* FST1.7, reaching 71.3 ± 0.0 mmol/L H^+. As a comparison, this same strain acidified mMRS up to 181 ± 5.4 mmol/L H^+ after 120 h. Although the mMRS was formulated without two buffering compounds (sodium acetate and potassium dihydrogen phosphate), the buffering capacity was still higher than in wort. This property of mMRS, together with the optimal nutrient composition of the substrate for LAB growth (Hébert et al., 2004), allowed for a higher accumulation of acids and a more moderate pH drop, ultimately leading to a lower extent of self-inhibition of the strains. The organic acid concentration and composition differed from strain to strain, with both obligate heterofermentative

Lb. brevis cultures releasing lower amounts of total acids and a higher acetic to lactic acid ratio (1:3 to 1:4 in wort) than the facultative heterofermentative *Lb. plantarum* FST1.7 (1:13 in wort). No propionic acid was detected. The four-fold stronger buffering capacity of mMRS compared to wort led to the higher accumulation of acids and to a more moderate pH drop. Maltose was present at high concentrations in unfermented wort (32.24 g/L) and was the preferred sugar consumed by both *Lb. brevis* strains, whereas *Lb. plantarum* FST1.7 fed mainly on the monosaccharides, glucose and fructose. Ethanol was released as part of the heterofermentative metabolism of the strains, reaching a concentration of 0.62 g/L in wort.

From a total of fifteen phenolic acids analysed, five were present in unfermented wort (4-hydroxybenzoic acid, hydrocaffeic acid, vanillic acid, *p*-coumaric acid, and ferulic acid). 3-phenyllactic acid (3-PLA) was the only phenolic compound produced by each strain in both substrates, with *Lb. plantarum* FST1.7 being the highest producer in wort (28.16 mg/L). Strain-dependent release of phenolic acids compounds consisted of phloretic (only in mMRS) and hydroferulic acid for *Lb. plantarum* FST1.7 and benzoic acid for both *Lb. brevis* strains (only in mMRS). Spore inhibition efficiency of the fermented worts varied significantly ($P < 0.05$) between the strains, with *Lb. brevis* L1105 accounting for the lowest inhibition (54 h), and *Lb. plantarum* FST1.7 for the highest (198 h). The latter also led to the longest inhibition in fermented mMRS (257 h).

Table 5. Initial and residual cell counts [log cfu/mL], pH, c[H^+] [mmol/L], sugars [g/L], ethanol [g/L], organic acids [g/L], phenolic compounds [mg/L] and spore inhibition times [h] (against 4 log spores/mL of *F. culmorum*) detected in mMRS and wort 6% (w/w) after 48 h fermentation with *Lb. brevis* L1105, *Lb. brevis* R2Δ and *Lb. plantarum* FST1.7 and compared to the control substrates.

	Substrate Control		*Lb. brevis* L1105		*Lb. brevis* R2Δ		*Lb. plantarum* FST1.7	
	mMRS	Wort 6%	mMRS	Wort 6%	mMRS	Wort 6%	mMRS	Wort 6%
Cell counts	< 1	< 1	9.12 ± 0.36	9.38 ± 0.18	9.66 ± 0.07	9.48 ± 0.06	10.88 ± 0.02	10.65 ± 0.05
pH	5.48 ± 0.02	5.78 ± 0.03	3.89 ± 0.06	3.57 ± 0.01	3.76 ± 0.01	3.55 ± 0.01	3.15 ± 0.01	3.04 ± 0.00
c[H^+]	21.1 ± 1.0	5.0 ± 0.0	48.3 ± 1.5	31.1 ± 0.5	66.5 ± 1.5	39.7 ± 0.5	139.7 ± 3.9	53.3 ± 0.8
Glucose	21.74 ± 0.04	5.46 ± 0.05	11.09 ± 0.34	4.34 ± 0.10	5.71 ± 0.08	4.39 ± 0.05	3.34 ± 0.03	0.61 ± 0.12
Fructose	-	1.39 ± 0.01	-	1.40 ± 0.05	-	0.94 ± 0.01	-	0.53 ± 0.07
Maltose	0.29 ± 0.05	32.24 ± 0.22	0.30 ± 0.05	25.89 ± 0.49	0.27 ± 0.01	23.23 ± 0.25	-	27.69 ± 1.53
Maltrotriose	-	6.39 ± 0.02	-	6.05 ± 0.09	-	6.18 ± 0.04	-	6.85 ± 0.38
Ethanol	-	-	0.49 ± 0.05	0.51 ± 0.03	0.77 ± 0.02	0.62 ± 0.06	-	0.24 ± 0.04
Lactic acid	-	-	1.86 ± 0.02	1.14 ± 0.03	2.64 ± 0.06	1.62 ± 0.11	9.59 ± 0.15	2.78 ± 0.08
Acetic acid	-	-	0.35 ± 0.03	0.27 ± 0.09	0.52 ± 0.03	0.47 ± 0.03	0.17 ± 0.00	0.21 ± 0.01
4-Hydroxybenzoic acid	-	0.28 ± 0.03	-	0.34 ± 0.13	-	0.30 ± 0.01	9.72 ± 1.68	1.05 ± 0.27
Hydrocaffeic acid	-	4.20 ± 0.09	-	2.61 ± 0.14	-	3.59 ± 0.25	-	4.15 ± 0.65
Vanillic acid	-	0.68 ± 0.03	-	0.60 ± 0.10	-	0.49 ± 0.07	-	0.33 ± 0.25
Phloretic acid	-	-	-	-	-	-	1.69 ± 0.17	-
3-Phenyllactic acid	-	-	4.20 ± 0.52	1.39 ± 0.33	13.35 ± 1.17	2.02 ± 0.05	11.30 ± 2.00	28.16 ± 2.28
Hydroferulic acid	-	-	-	-	-	-	4.03 ± 0.38	3.05 ± 0.29
p-Coumaric acid	-	1.46 ± 0.10	-	1.27 ± 0.07	-	1.20 ± 0.08	-	-
Ferulic acid	-	2.72 ± 0.12	-	1.98 ± 0.36	-	2.61 ± 0.11	-	-
Benzoic acid	-	-	5.67 ± 0.92	-	6.85 ± 0.09	-	-	-
Spore inhibition	11 ± 3	9 ± 0	45 ± 3	54 ± 2	76 ± 2	95 ± 9	257 ± 15	198 ± 19

Each value was expressed as mean ± standard deviation analysed from three independent fermentations.

3.4.3 Effect of fermentation conditions on antifungal activity of wort

Fermentation of wort of increasing malt extract (from 3% (w/w) to 12% (w/w)) resulted in a significant ($P < 0.05$) accumulation of 3-PLA, hydroferulic and lactic acid concentrations as well as longer inhibition of spore outgrowth (Table 6). A maximum of up to 11 days inhibition was found for the 12% (w/w) wort fermented by *Lb. plantarum* FST1.7. Wort fermented at a higher temperature (37°C) than the optimal one (30°C) slightly decreased the amount of phenolic compounds, and the period of fungal inhibition was reduced to up to the half of the original time for both *Lb. brevis* strains. Growth for both *Lb. brevis* strains was sub-optimal at 37°C when compared to *Lb. plantarum* FST1.7. Significantly lower levels of organic acid production at 37°C were only detected for strain R2Δ. Finally, longer incubation times (from 24 h to 120 h) at 30°C allowed for prolonged matrix acidification, mainly in form of lactic and acetic acid. Only *Lb. plantarum* FST1.7 released higher amounts of phenolic compounds (hydroxybenzoic and 3-phenyllactic acid) with longer fermentation (Table 6).

3.4.4 MIC in malt extract-based substrate against *F. culmorum*

Malt extract broth (MEB) was used as substrate for MIC assessment, as no phenolic compounds could be detected in this medium ($n = 3$). The MIC values found for the single compounds, as outlined in Table 7, were generally much higher than the concentrations found in the fermented media. The MIC range varied significantly between compounds and ranged from 125 mg/L for benzoic acid to 6000 mg/L for hydrocaffeic acid. The pH adjustment to 3.00 ± 0.05 significantly decreased the MIC for the single compounds to up to a fourth of their former amount. Ethanol proved to be a rather ineffective agent against *Fusarium* spores outgrowth, showing the highest MIC (26000 mg/L) among the metabolites tested.

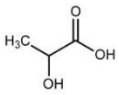
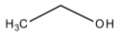
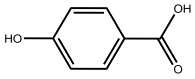
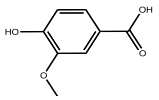
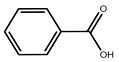
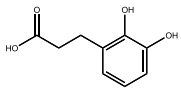
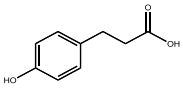
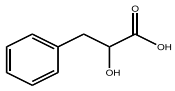
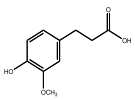
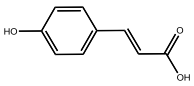
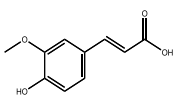
Table 6. Quantification of antifungal compounds released from LAB in wort under different fermentation conditions and spores inhibition values (against 4 log spores/mL of *F. culmorum*).

Variable condition	4-Hydroxybenzoic acid [mg/L]	3-Phenyllactic acid [mg/L]	Hydroferulic acid [mg/L]	Lactic acid [g/L]	Acetic acid [g/L]	Spores inhibition [h]
Extract content (% w/w) (30°C, 48 h)						
Lb. brevis L1105						
- 3%	-	0.73 ± 0.19 ^{c}	-	0.83 ± 0.01 ^{d}	0.26 ± 0.02 ^{a}	45 ± 2 ^{b}
- 6%	-	1.40 ± 0.33 ^{c}	-	1.14 ± 0.03 ^{c}	0.36 ± 0.11 ^{a}	54 ± 2 ^{b}
- 9%	-	2.67 ± 0.46 ^{b}	-	1.39 ± 0.01 ^{b}	0.39 ± 0.01 ^{a}	67 ± 7 ^{b}
- 12%	-	3.54 ± 0.28 ^{a}	-	1.56 ± 0.04 ^{a}	0.32 ± 0.00 ^{a}	153 ± 29 ^{a}
Lb. brevis R2Δ						
- 3%	-	1.09 ± 0.12 ^{d}	-	1.53 ± 0.04 ^{b}	0.36 ± 0.03 ^{d}	79 ± 3 ^{c}
- 6%	-	2.02 ± 0.05 ^{c}	-	1.62 ± 0.04 ^{b}	0.47 ± 0.01 ^{c}	95 ± 9 ^{bc}
- 9%	-	2.77 ± 0.10 ^{b}	-	1.75 ± 0.05 ^{ab}	0.61 ± 0.01 ^{b}	101 ± 4 ^{b}
- 12%	-	4.69 ± 0.19 ^{a}	-	1.83 ± 0.02 ^{a}	0.74 ± 0.01 ^{a}	186 ± 14 ^{a}
Lb. plantarum FST1.7						
- 3%	1.14 ± 0.33 ^{a}	22.03 ± 0.52 ^{d}	1.75 ± 0.31 ^{d}	2.68 ± 0.19 ^{b}	0.24 ± 0.04 ^{a}	156 ± 5 ^{c}
- 6%	1.05 ± 0.27 ^{a}	28.16 ± 2.28 ^{c}	3.05 ± 0.29 ^{c}	2.78 ± 0.08 ^{b}	0.21 ± 0.01 ^{a}	198 ± 19 ^{b}
- 9%	0.91 ± 0.17 ^{a}	36.64 ± 2.11 ^{b}	4.87 ± 0.49 ^{d}	2.86 ± 0.11 ^{b}	0.25 ± 0.01 ^{a}	221 ± 7 ^{b}
- 12%	1.16 ± 0.12 ^{a}	41.77 ± 1.15 ^{a}	6.74 ± 0.58 ^{a}	3.25 ± 0.01 ^{a}	0.23 ± 0.02 ^{a}	267 ± 10 ^{a}
Temperature (*6% (w/w)*, 48 h)						
Lb. brevis L1105						
- 30°C	-	1.40 ± 0.33 ^{a}	-	1.14 ± 0.03 ^{a}	0.36 ± 0.11 ^{a}	54 ± 2 ^{a}
- 37°C	-	1.79 ± 0.10 ^{a}	-	1.02 ± 0.14 ^{a}	0.23 ± 0.06 ^{a}	25 ± 2 ^{b}

Lb. brevis R2Δ						
- 30°C	-	2.02 ± 0.05 [a]	-	1.62 ± 0.04 [a]	0.47 ± 0.01 [a]	95 ± 9 [a]
- 37°C	-	1.50 ± 0.32 [a]	-	1.33 ± 0.07 [b]	0.34 ± 0.01 [b]	45 ± 7 [b]
Lb. plantarum FST1.7						
- 30°C	1.05 ± 0.27 [a]	28.16 ± 2.28 [a]	3.05 ± 0.29 [a]	2.78 ± 0.08 [a]	0.21 ± 0.01 [a]	198 ± 19 [a]
- 37°C	1.08 ± 0.04 [a]	25.46 ± 3.44 [a]	3.23 ± 0.31 [a]	2.92 ± 0.13 [a]	0.22 ± 0.01 [a]	190 ± 6 [a]
Time (*6% (w/w)*, 30°C)						
Lb. brevis L1105						
- 24 h	-	1.02 ± 0.12 [a]	-	0.77 ± 0.13 [c]	0.19 ± 0.02 [b]	50 ± 2 [a]
- 48 h	-	1.40 ± 0.33 [a]	-	1.14 ± 0.03 [c]	0.36 ± 0.11 [ab]	54 ± 2 [a]
- 72 h	-	1.38 ± 0.06 [a]	-	1.58 ± 0.07 [b]	0.36 ± 0.04 [ab]	52 ± 1 [a]
- 120 h	-	1.27 ± 0.08 [a]	-	1.71 ± 0.12 [a]	0.45 ± 0.04 [a]	74 ± 17 [a]
Lb. brevis R2Δ						
- 24 h	-	1.63 ± 0.04 [b]	-	1.19 ± 0.09 [c]	0.24 ± 0.04 [c]	58 ± 1 [b]
- 48 h	-	2.02 ± 0.05 [a]	-	1.62 ± 0.04 [b]	0.47 ± 0.01 [b]	95 ± 9 [a]
- 72 h	-	2.05 ± 0.04 [a]	-	1.93 ± 0.06 [a]	0.56 ± 0.06 [ab]	98 ± 2 [a]
- 120 h	-	1.98 ± 0.07 [a]	-	2.23 ± 0.14 [a]	0.63 ± 0.03 [a]	113 ± 15 [a]
Lb. plantarum FST1.7						
- 24 h	0.55 ± 0.05 [b]	14.72 ± 0.50 [c]	2.21 ± 0.23 [b]	1.77 ± 0.07 [c]	0.09 ± 0.06 [b]	74 ± 4 [b]
- 48 h	1.05 ± 0.27 [a]	28.16 ± 2.28 [b]	3.05 ± 0.29 [a]	2.78 ± 0.08 [b]	0.21 ± 0.01 [b]	198 ± 19 [a]
- 72 h	1.18 ± 0.12 [a]	36.00 ± 1.03 [a]	2.56 ± 0.50 [ab]	3.14 ± 0.35 [ab]	0.35 ± 0.06 [a]	198 ± 32 [a]
- 120 h	1.41 ± 0.04 [a]	38.42 ± 1.32 [a]	2.39 ± 0.12 [b]	3.75 ± 0.45 [a]	0.45 ± 0.06 [a]	232 ± 7 [a]

Each value was expressed as mean ± standard deviation analysed from three independent fermentations.
Inhibition time for unfermented control substrates (wort at 3, 6, 9, 12% (w/w)) at 30°C and 37°C amounted to 10 ± 2 h.
a-d For each compound and fermentation variable, a different superscript in each column denotes a significant difference at $P < 0.05$.

Table 7. Minimal inhibitory concentration MIC [mg/L] of wort-own, LAB-released antifungal compounds against 4 log spores/mL of *F. culmorum* in MEB with and without pH correction compared to the highest values [mg/L] found after wort fermentation in this study.

Metabolite	Chemical structure	pKa	MIC *	MIC (pH 3.00 ± 0.05)	c_{max}
Lactic acid		3.86	7000 (2.54)	8000	3750
Acetic acid		4.76	1000 (3.85)	250	740
Ethanol		-	26000 (5.02)	22000	620
Hydroxybenzoic acids (C_6-C_1)					
4-Hydroxybenzoic acid		4.54	2500 (3.59)	1500	1.16
Vanillic acid		4.16	1500 (3.83)	750	1.12
Benzoic acid		4.20	125 (4.69)	31.25	-
Hydroxycinnamic acids (C_6-C_3)					
Hydrocaffeic acid		3.84	6000 (3.52)	6000	8.74
Phloretic acid		4.21	2500 (3.78)	1500	-
3-Phenyllactic acid		3.46	2000 (3.93)	1500	41.77
Hydroferulic acid (dihydroferulic acid)		3.37	2000 (3.98)	1000	6.74
p-Coumaric acid		4.00	750 (4.20)	500	2.02
Ferulic acid		4.58	400 (4.40)	300	6.33

Each MIC value was calculated from three independent experiments.

* pH value of the MEB substrate with phenolic acid.

3.4.5 Spiking tests in MEB and wort substrates against *F. culmorum*

The spiking results showed that the addition of the phenolic compounds in unfermented wort and MEB substrate at levels found after fermentation of wort did not significantly increase inhibition (Table 8). However, pH adjustment of unfermented wort to the post-fermentation levels found for *Lb. plantarum* FST1.7 (pH 3.04) significantly retarded fungal growth (inhibition up to 18 h and 30 h in MEB and wort, respectively, compared to 9 h in both unfermented media). The subsequent addition of antifungal phenolic compounds in the pH-corrected substrates could prolong inhibition by 2 h for *Lb. brevis* R2Δ to up to 10 h for *Lb. plantarum* FST1.7, even though these increases were not statistically significant. The addition of organic acids with pH correction contributed to the strongest antagonistic effect on fungal spores. For *Lb. brevis* R2Δ and *Lb. plantarum* FST1.7, this led to an additional delay in spore outgrowth of 38 h (+200%) and 24 h (+133%) in MEB, and of 72 h (342%) and 28 h (93%) in wort, respectively, when compared to the pH-corrected control substrates. The final addition of phenolic compounds to the pH-corrected substrates containing the organic acids had little to no effect on fungal inhibition for *Lb. brevis* R2Δ, but significantly increased inhibition by *Lb. plantarum* FST1.7, resulting in 21 h and 18 h longer fungal inhibition in MEB and wort, respectively.

Table 8. Inhibition time [h] of MEB and wort 6% (w/w) substrates with added phenolic acids (PA), organic acids (OA) and pH adjustment found after fermentation of wort 6% for *Lb. brevis* R2Δ and *Lb. plantarum* FST1.7 (against 4 log spores/mL of *F. culmorum*).

Mix	**MEB**	**Wort**
***Lb. brevis* R2Δ**	**Spores inhibition**	**Spores inhibition**
Control pH 5.43 in MEB; pH 5.78 in wort	9 ± 3 ^{c}	9 ± 0 ^{c}
PA pH 5.38 in MEB; pH 5.74 in wort	9 ± 2 ^{c}	10 ± 1 ^{c}
pH 3.55	19 ± 1 ^{b}	21 ± 1 ^{b}
PA (pH 3.55)	21 ± 0 ^{b}	23 ± 2 ^{b}
OA (pH 3.55) AA: 470 mg/L, LA: 1620 mg/L	57 ± 1 ^{a}	93 ± 8 ^{a}
PA / OA (pH 3.55)	61 ± 12 ^{a}	98 ± 17 ^{a}
***Lb. plantarum* FST1.7**	**Spores inhibition**	**Spores inhibition**
Control pH 5.43 in MEB; pH 5.78 in wort	9 ± 3 ^{d}	9 ± 0 ^{d}
PA pH 5.42 in MEB; pH 5.60 in wort	10 ± 2 ^{cd}	14 ± 1 ^{d}
pH 3.04	18 ± 4 ^{cd}	30 ± 1 ^{c}
PA (pH 3.04)	21 ± 1 ^{c}	40 ± 2 ^{c}
OA (pH 3.04) AA: 210 mg/L, LA: 2780 mg/L	42 ± 3 ^{b}	58 ± 8 ^{b}
PA / OA (pH 3.04)	63 ± 4 ^{a}	76 ± 2 ^{a}

Each value was calculated from three independent experiments.
AA: acetic acid; LA: lactic acid.
a-d For each substrate and spiked conditions, a different superscript in each column denotes a significant difference at $P < 0.05$.

3.5 Discussion

Barley malt extract is an attractive substrate for LAB fermentation and antifungal enrichment because of its inexpensive nature and ease of application in food-grade systems, without the need for laborious purification steps. The primary carbon and energy source was maltose, followed by glucose. The consumption patterns suggested that the three strains were able to metabolise both sugars; however, species-specific metabolism and preferences for one or the other sugar influenced the type and amount of organic acids produced, which in turn determined the final antifungal strength of the ferments. Because of the lower MIC of acetic acid against *F. culmorum* macroconidia compared to lactic acid (which was seven times higher in MEB at pH 3), it is encouraging to use starter cultures that are able to enrich acetic acid over lactic acid, to levels that do not negatively impact organoleptic properties (Engan, 1974). Nevertheless, lactic acid, as the quantitatively most relevant metabolite released during LAB fermentation, played a fundamental role in the final fungal inhibition by decreasing the pH of the medium. The importance of a pH-mediated antifungal effect was confirmed by Gerez et al. (2013), who found that the majority of the LAB strains tested against food spoiling fungi had significantly decreased (40-80%) their antifungal efficiency after pH-neutralisation of their cell-free supernatants. According to the ionisation constant, pKa, of carboxylic acids (Lind et al., 2005; Oliveira et al., 2014), compounds with higher pKa values, e.g. acetic acid or ferulic acid, will mainly be in their protonated form under the acidic wort environment compared to compounds with lower pKa values. This improves their diffusion across the hydrophobic fungal membrane with subsequent dissociation. However, the ionisation constant does not fully explain the lower MIC values found for certain phenolic acids, e.g. *p*-coumaric acid (pKa 4.00 and MIC of 500 mg/L) compared to vanillic acid (pKa 4.21 and MIC of 1500 mg/L) (Table 7). Unsaturated side chains can impart an additional hydrophobic character to the compounds. This was confirmed in our study by the low MIC levels found for *p*-coumaric and ferulic acid (500 and 300 mg/L, respectively), both containing a double bond in their hydrocarbon side chains. Furthermore, Sánchez-Maldonado et al. (2011) found that the antibacterial activity of various phenolic acids, other than increasing with lower pH, negatively correlated with the amount of hydroxyl groups carried by the compound. This would explain the high MIC found for hydrocaffeic acid (ring substituted with two hydroxyl groups) when compared to the lowest found for benzoic acid (no ring substitution). Benzoic acid, as one of the metabolite of certain

species of LAB, was linked to the high antifungal efficiency of *Lb. reuteri* R29 against *F. culmorum* in a wort-based freeze-dried substrate (Oliveira et al., 2014). Ethanol, as the only non-acid antifungal compound analysed, showed little pH dependency. Past studies also confirmed that little interaction occurs between ethanol and organic acids (Oh and Marshall, 1993).

Similar to the majority of the other phenolic compounds, 3-PLA can be formed by LAB through amino acid (phenylalanine) catabolism (Li et al., 2007). 3-PLA is a promising antifungal compound due to its non-toxicity on human and cell lines (Oberdoerster et al., 2000) and its odourless nature compared to other metabolites, e.g. acetic acid. This compound was the only one produced by all LAB cultures tested in this study; however, concentrations varied between strains, and more remarkably, between species. Differences in metabolism together with a better adaptation to the substrate could possibly explain the significantly higher concentration of 3-PLA released by *Lb. plantarum* FST1.7 (up to fourteen times higher) compared to *Lb. brevis* R2Δ. Nevertheless, when the compound was tested in acidic wort at the concentration found (28.16 mg/L), no prolonged inhibition was detected. Only when this amount of 3-PLA was mixed together with hydroferulic acid (3.05 mg/L) in acidic wort, they could decrease spore outgrowth, showing synergies at levels far below their MIC (1500 mg/L for 3-PLA and 1000 mg/L for hydroferulic acid at pH 3). High concentrations (188 mg/L) of 3-PLA after lactic fermentation in wort were found by Oliveira et al. (2014) using *Lb. reuteri* R29. It was hypothesised that the proteolytic activity of this strain could have increased the pool of amino acids acting as precursors for 3-PLA formation (Axel et al., 2016).

Phenolic compounds are naturally present as secondary metabolites in the barley husk (Vogel, 2008) and serve as microbial and/or endogenous growth regulators (Briggs et al., 1981). In this study, 4-hydroxybenzoic, hydrocaffeic, vanillic, *p*-coumaric and ferulic acids were found to be present in the unfermented wort. The same compounds have been found to be actively released by *Lb. plantarum* strains inoculated on grass silage (Broberg et al., 2007). At the very low concentrations found in the present study, they did not contribute to any noticeable increase in the fungistatic effect when tested as a mixture in MEB. Nevertheless, the low MIC values of vanillic, ferulic and *p*-coumaric acids support strategies that aim to enrich such compounds. The use of enzyme preparations, e.g. α-amylase and cellulase (Yu et al., 2001) or feruoyl esterase (Faulds et al., 2002; Szwajgier, 2011) have been shown to improve the release of bound phenolic

compounds in cereal substrates. Added proteases or cultures with proteolytic activities could be employed to increase the release of amino acids, precursors of phenolic acids produced by LAB. This would also increase the buffering capacity of the wort and possibly lead to an extended acidification period. Furthermore, it is well known that certain LAB strains can metabolise some of these compounds via decarboxylation and/or reduction reactions (Van Beek and Priest, 2000) to form other phenolic compounds. The reduction of *p*-coumaric acid to phloretic acid (Sánchez-Maldonado et al., 2011), and ferulic acid into hydroferulic acid (Svensson et al., 2010), was shown only for *Lb. plantarum* FST1.7. The potential for such metabolic conversions should be taken into account during strain selection, as this can produce phenolic acids with higher MIC values and lower potency.

Increase in the wort extract had a positive impact on both the production of organic and phenolic acids, especially 3-PLA. This hints to the deficiency of essential nutrients needed for the metabolism of phenolic acids, i.e. amino acids, in the more dilute worts. On the other hand, longer fermentations had less effect on phenolic acid release, but led to a progressive accumulation of organic acids and thus, lower pH levels. It was found that the antifungal efficiency of the fermented wort significantly increased ($P < 0.05$) between 24 h and 48 h of fermentation, corresponding to the stationary phase of LAB growth, before reaching a plateau. Comparing the antifungal impact of fermented worts, fermentation of 9% (w/w) wort for 48 h delivered a similar inhibition effect as one performed in 6% wort for 72 h. However, the 3% wort fermented for 48 h was significantly more fungistatic that 6% wort fermented for 24 h. The timing of antifungal compounds release has important implications for *in situ* food applications, such as during malting, when both the LAB starter cultures and their fungal antagonists are simultaneously present on the cereal kernels and compete for the same resources.

The LAB strains examined in this study have an optimal growth temperature of around 30°C (77). Mauch et al. (2010) found that the MRS-based cfs for some strains of *Lb. brevis* led to better inhibition of *Fusarium* species after incubation of the strains at higher temperatures (37°C). However, an improvement of antifungal activity due to a stress-induced change of fermentation temperature could not be detected in this study. Both *Lb. brevis* strains registered a 0.7 to 1.5 log drop in cell growth (after 48 h), partially explaining the lower accumulation of carboxylic acids and poorer results in spore inhibition compared to the cfs produced at 30°C.

Finally, the spiking experiments showed that the inhibition effect produced by mimicking the concentrations of phenolic acids, organic acids, and pH level, as found after wort fermentation, accounted for the entirety of the antifungal effect observed for *Lb. brevis* R2Δ. The low pH and the high concentration of acetic acid, close to its MIC, were possibly responsible for the majority of the inhibitory effect, with the phenolic acids playing a secondary role at the concentrations present. In contrast, the spiking experiments for *Lb. plantarum* FST1.7 could only explain 38% of the inhibition potential of the fermented wort. Other compounds not quantified in this study were most likely contributing to the fungal inhibition, such as diacetyl, often produced by *Lb. plantarum* species (Aunsbjerg et al., 2015), or compounds of a proteinaceous nature, e.g. peptide-based antifungals (Coda et al., 2011). Similar results were found in a wheat sourdough matrix, where phenolic acids had been spiked in the dough and found to increase the shelf-life compared to a chemically acidified dough (Axel et al., 2016). However, sourdough containing the highest concentration of these compounds did not lead to the longest growth inhibition against environmental moulds.

Industrial application of a LAB-fermented substrate for antifungal purposes could be particularly interesting for industries dealing with significant microbiological inconsistencies of their raw materials. In this regard, malting of cereals could be an excellent candidate to help control and standardise the natural microbiota on the surface of grain kernels. Similar to the production of acidified wort, produced for the purposes of biological acidification during mashing and boiling (Kunze, 2004), fermentation of malt extract could be done in temperature-controlled acidification vessels. According to the malting batch plan, the acidified substrate could be discontinuously withdrawn and directly applied on the surface of the grain kernels, while tanks are being refilled with fresh substrate and fed again to the LAB cultures. In this regard, high LAB cell concentrations would help to quickly dominate fermentation after industrial handling of the unfermented substrate. On the perspective of cost optimisation, such a process could serve as an outlet for low grade malt (e.g. broken kernels), which could be used as a substrate (MEBAK, 2011). Final application of the antifungal ingredient could be done either by adding into the steeping water or by spraying on the kernels during the germination process (Kunze, 2004). According to previous studies, steeping of LAB solutions has been preferred over spraying because of better consistency in results (Laitila et al., 2006; Mauch et al., 2011).

3.6 Conclusion

This study examined the production and variety of carboxylic acids released by LAB cultures during wort fermentation. The final spore inhibition capacity of the fermented wort could be primarily attributed to organic acids, and to a lesser extent, phenolic compounds. The latter showed, at the concentrations released, only weak synergies with the pH level and organic acids. Good adaptability to the wort substrate by the LAB culture, long fermentation times, high extract content and buffering capacities of the wort medium could maximise the mixed accumulation of organic acids, phenolic acids and lead to an overall prolongation of the inhibitory period.

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3.8 References

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Chapter 4: *Lactobacillus brevis* R2Δ as starter culture to improve biological and technological qualities of barley malt

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4.1 Abstract

The application of lactic acid bacteria (LAB) can be a challenging yet promising tool to control the indigenous microbiota during malting and to improve malt quality. In this study, a food-grade malt-based ingredient was fermented using an antifungal strain, *Lactobacillus brevis* R2Δ, and applied on barley grains during steeping and germination. Different variations of starter culture concentration and cell free supernatant were compared to a control solution during pilot-scale malting trials. All treated barley samples showed a significant decrease in aerobic bacteria (up to 99% reduction) and a promotion of yeast growth when compared to the untreated control. The number of kernels contaminated with *Fusarium* spp. could be reduced by more than 90%, as confirmed by qPCR analysis. Shorter rootlets coincided with lower malting losses (-31.8%) and with increased extract yield (+3.1%). Differences in the enzymatic activity between the malts did not significantly alter the processability of the malts during brewhouse operations. Throughout yeast fermentation, no negative impact of LAB could be detected for the majority of attributes tested. Overall, the treatment containing living starter cultures and the highest amount of total titratable acidity (71 mmol/L) showed the most promising results when aimed at further enhancing the quality and safety of barley malt.

4.2 Introduction

The type and extent of metabolic changes taking place within a barley kernel during malting define the final malt quality and processability during brewing (Kunze, 2010). These are largely catalysed by the endogenous enzymes already present or newly formed during the malting process. However, microorganisms that colonise the grain surface also represent an important variable capable of influencing a kernel performance. The grain microbiota is highly dependent on pre- and post-harvesting factors, which determine its final composition (Justé et al., 2011; Justé et al., 2014). For this reason, crop batches can significantly differ from each other, leading eventually to inconsistent outcomes after malting. Bacteria, yeasts and filamentous fungi, the main member of the grain microbiota, have been linked to both beneficial and harmful effects on the final malt quality (Booysen et al., 2002; Doran and Briggs, 1993; Justé et al., 2011; Noots et al., 1999), thus the interest in controlling and managing these organisms.

A variety of chemical agents have been used in the past, e.g. potassium bromate, or are currently allowed to improve yield from barley to malt, e.g. gibberellic acid (9), but their use is not without health-related controversies (Kurokawa et al., 1990). Moreover, current trends such as "green label" and "free from", which promote a more natural approach to processing and preservation (Pawlowska et al., 2012), cannot be ignored by maltsters and brewers. In the past, starter cultures of both fungal and bacterial nature have been tested during malting with the aim of controlling fungal infection (Laitila et al., 2006; Laitila et al., 2007; Oliveira et al., 2015) and improving specific malt characteristics (Lowe et al., 2005a; Mauch et al., 2011a). Among the candidates used in these studies, lactic acid bacteria (LAB) have received special attention because of the mild but effective consequences that acidification has on both biological and process-technical attributes (Lowe and Arendt, 2004). Specifically, *Lactobacillus* starter cultures have been linked with decreased malting losses during malting (Mauch et al., 2011a; Schehl et al., 2007) and better filterability during brewing (Lowe et al., 2005a) when applied during malting. A mould-suppressing effect of several LAB strains has been confirmed during both *in vitro* and pilot scale malting of kernels artificially infected with *Fusarium* spp. (Laitila et al., 2002; Oliveira et al., 2014; Oliveira et al., 2015). Fusaria moulds comprise of diverse species able to release mycotoxins and are responsible for severe technological failures in beer, e.g. gushing (Amaha and Kitabatake, 1981). Finally, naturally present aerobic heterotrophic bacteria can also be critical as they can compete with the grain for oxygen (Van Campenhout et al., 1998; Doran and Briggs, 1993) and

release metabolites that eventually lead to germination delays (Bol et al., 1985) or complications during lautering (Kreisz et al., 2001).

The vast majority of studies aimed at reducing fungal load have relied on the application of functional LAB ferments composed of nutritionally rich, synthetic broths (Laitila et al., 2002; Laitila et al., 2006; Lowe et al., 2005a; Mauch et al., 2011a). However, these media are unsuitable for direct application in food processing operations, unless the active compounds have been previously purified. The attractiveness of an alternative ingredient relies on the direct application to the barley surface, avoiding expensive down-stream purification costs. As a cheap and food-grade alternative, barley malt extract (or wort) is an ideal substrate due to the potential for acceptance and accessibility by both maltsters and brewers. It has been shown that wort is a suitable substrate for LAB growth as well as to enrich antifungal metabolites (Oliveira et al., 2014; Peyer et al., 2016). Similar to the industrial production of acid malt, during which barley malt is being sprayed or soaked in a wort solution previously fermented by LAB strains (Kunze, 2010), the same approach can be adopted on unmalted barley before and during the malting process. Oliveria et al. (2015) showed that the application of a cell-free solution based on LAB fermented wort could control fungal spread (up to 23% reduction) on grains artificially infected with *Fusarium culmorum*. While the majority of studies examined the application of a cell-free spent medium, others have emphasised the beneficial use of whole cell cultures (cells and spent medium) in order to benefit from both the antimicrobial compounds released in the culture broth and the continued growth and action *in situ* of the strain (Haikara et al., 1993; Laitila et al., 2002). At present, more knowledge is still needed to understand which combination of spent medium and living starter culture provides the most efficient bioprotection in addition to improved malt quality.

This research aims to build on previous studies to further understand LAB as bioprotective starter cultures and as a technological aid when applied during malting of barley. In this regard, *Lb. brevis* R2Δ was chosen as starter culture because of its ability to release active antifungal compounds, such as carboxylic and phenolic acids as well as cyclic dipeptides (Axel et al., 2014), and its demonstrated ability to efficiently inhibit *F. culmorum* when tested *in vitro* using spent diluted wort (6% (w/w)) (Peyer et al., 2016). Treatments characterised by different cell concentrations and spent culture media were applied during steeping and germination of the kernels, when grain properties and

microflora development are highly transitional and easier to influence. The final malts were further assessed for relevant brewing qualities.

4.3 Materials and methods

4.3.1 Cultures and ingredients

The strain *Lb. brevis* R2Δ (culture collection of the Cereal and Beverages Research Group, University College Cork, Ireland) was routinely propagated on deMan Rogosa Sharp (MRS) (Sigma-Aldrich, St. Louis, USA) plates for isolation of single colonies for further propagation. *F. culmorum* TMW 4.2043 (isolate from barley) and *F. graminearum* DSM 4528 (isolate from maize) were provided by the culture collection of TU München (Weihenstephan, Germany) and by the German Collection of Microorganisms and Cell Culture (DSMZ, Braunschweig, Germany), respectively. Commercial spring malting barley (*Hordeum vulgare,* Beatrix variety), harvested in 2014, was obtained from Saaten-Union (Estrees Saint Denis, France). Analysis on the barley quality showed that this was of good brewing quality according to the guidelines of the Mitteleuropäische Brautechnische Analysenkommission (MEBAK, 2011a; MEBAK, 2011b). All reagents used in the following trials were at least analytical grade from Sigma-Aldrich (St. Louis, USA) unless otherwise stated.

4.3.2 Steeping and germination solutions

Steeping (SS) and germination (GS) solutions were freshly prepared for immediate use during malting. Single colonies of the starter culture were propagated in 45 mL of liquid MRS broth (24 h, 30°C), washed twice with Ringer's solution and propagated twice in 3% (w/w) sterile, unhopped wort produced from spray-dried malt extract (Muntons plc, Stowmarket, UK) (Figure 5). The control treatment (C-T) was performed using water, while addition of lactic and acetic acid to the levels found in the fermented wort treatment (FW-T) was done for the chemically acidified control treatment (CA-T). All solutions were chilled to the steeping and germinating temperature of 14°C before application to barley. The final attributes of each solution are reported in Table 9.

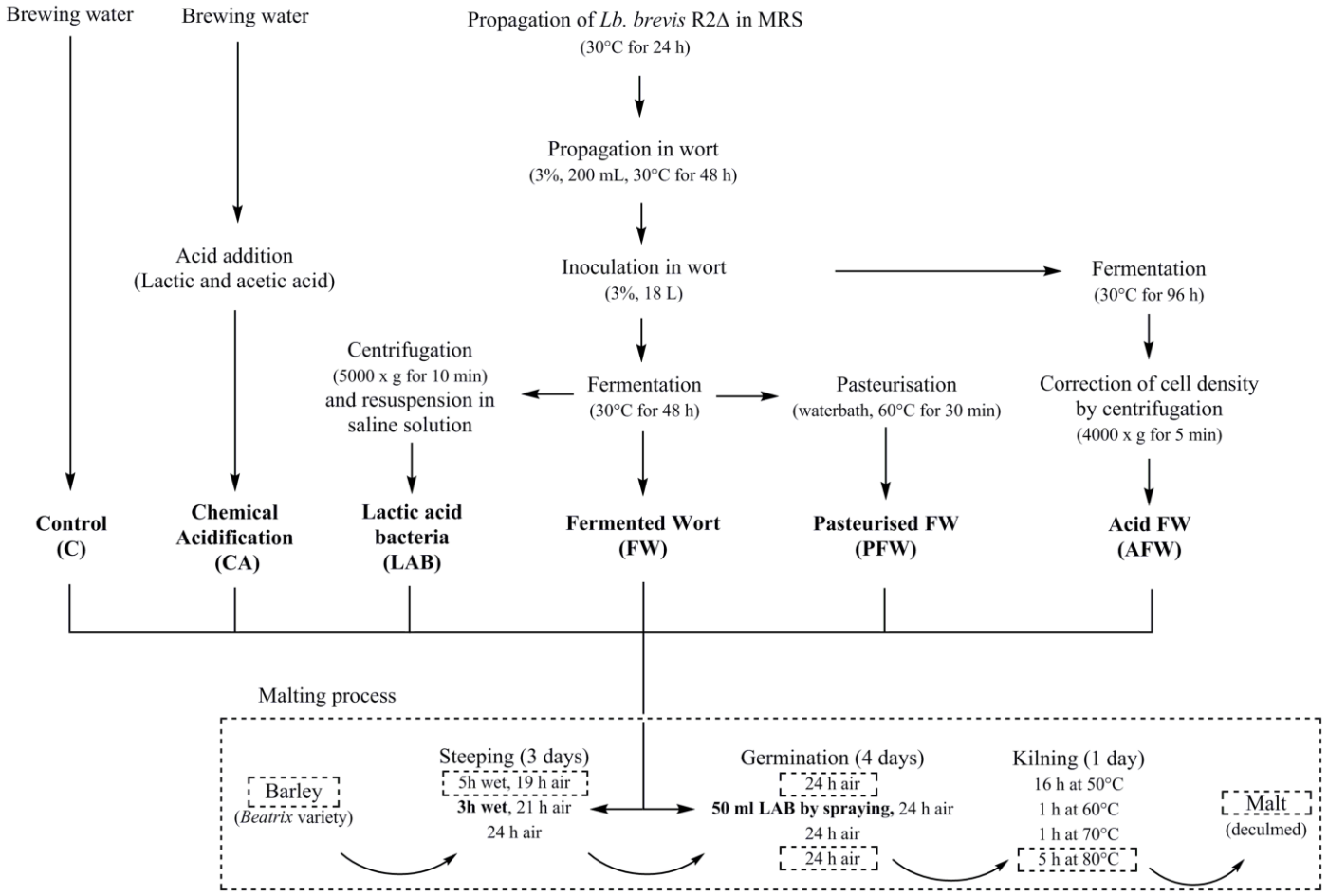

Figure 5. Flow diagram of *Lb. brevis* R2Δ propagation and treatment preparation for application during steeping and germination of barley grains. Framed steps during malting indicate sampling times.

Table 9. Cell counts [log cfu/mL], pH and titratable acidity [mmol/L] of the steeping and germination solutions.

Treatment	**Control**	**Chemical Acidification**	**Lactic acid bacteria**	**Fermented wort (FW)**	**Pasteurised FW**	**Acid FW**
Code	**C**	**CA**	**LAB**	**FW**	**PFW**	**AFW**
Cell counts [log cfu/mL]	< 1	< 1	9.9 ± 1.9	10.2 ± 0.3	1.4 ± 0.2	8.4 ± 0.8
pH [-]	7.02 ± 0.05	2.77 ± 0.00	6.39 ± 0.13	3.76 ± 0.14	3.46 ± 0.01	3.04 ± 0.00
$C[H^+]$ [mmol/L]	< 1.0	21.0 ± 0.0	< 1.0	20.0 ± 1.4	34.5 ± 0.7	71.0 ± 0.0

4.3.3 Application of the solutions during malting

Malting trials were carried out following a modified standard micro-malting procedure (Figure 5) according to MEBAK Method 1.5.3 (MEBAK, 2011a). Eight perforated stainless-steel boxes were each filled with 1000.0 g raw barley and placed in a stainless-steel vat filled with 250 L of water maintained at 14°C by automatic glycol-cooling during steeping for the first steeping step. Air and germination steps were carried out in a humidity control chamber (98% specific humidity, 14°C) (KOMA Koeltechnische Industrie B.V., Roermond, Netherlands). Steeping boxes were sealed at the bottom with tape and the grain samples were individually steeped with 2 L of each treatment solution for three hours before the second air rest. Throughout germination, the water content was corrected to 45.0% every 24 h by spraying water while mixing the grains thoroughly, thus preventing clump formation. A second treatment application was performed on the second day of germination by spraying 50 mL of the respective solutions on each tray. The grains were photographed each day to follow the growth process of the kernels. Finally, the green malt was kilned using a micromalting machine (Joe White Malting Systems, Melbourne, Australia). Each tray was treated as an individual malting experiment. Removal of the rootlet from the kilned malt was done using a thresher (LD180, Wintersteiger AG, Ried, Austria). Samples of grains were taken regularly throughout the malting process and directly investigated for culture-dependent microbiological analysis, while the rest was stored at -20°C for other analysis.

4.3.4 Microbiological counts during malting

Plate counts were prepared by randomly sampling thirty grains and horizontally shaking (Vibrax VXR basic, IKA-Werke GmbH & CO. KG, Staufen, Germany) these in 6 mL of Ringer's solution at 1000 rpm for 4 h. The supernatants were serially diluted (10^{-1} to 10^{-8}) and viable cell counts of LAB, aerobic heterotrophic bacteria and yeasts was done on selective agar plates according to Laitila et al. (2006). For the qualitative detection of fusaria growth, the Czapek-Dox selective agar medium containing Iprodione and Dicloran (CZID) was used as described by Abildgren et al. (1987). For this purpose, 15 kernels were placed on CZID-plates and red fungal growth was assessed after 5 days of incubation at room temperature. The quantification of reddish malt kernels was visually determined by counting the grains with visible reddish coloration in 200 g of malt (MEBAK, 2011a). In this regard, partially red kernels were also taken into account. All microbiological analyses were performed in triplicate.

4.3.5 qPCR of *F. culmorum* and *F. gramineraum*

A total of thirty malt kernels (duplicate for each treatment) were ground to flour by horizontal shaking using a chilled grinding jar (20 s at 30 Hz) (TissueLyser II, Qiagen, Hilden, Germany). From each pulverised sample, 250 mg were used for DNA extraction using the MoBio PowerSoil® DNA isolation kit (MoBio Laboratories, Inc. Solana Beach, USA) according to the manufacturer's instructions with slight modifications. The samples were bead-beated using glass beads (150-212 µm) (Sigma Aldrich) and a Ribolyser (MagNA Lyser, Roche Diagnostics GmbH, Mannheim, Germany) twice for 30 s at speed setting 6000, and 50 µL were used to elute the DNA. The extracted DNA was stored at -80°C until further use. As a positive control for the target fungi, *F. culmorum* and *F. graminearum* control strains were cultivated in 150 mL potato dextrose broth (PDB) (Fluka, Buchs, Switzerland). After 4 days incubation at 25°C under static conditions, the mycelium was collected on a sterile filter paper (Whatman 113, Maidstone, UK), and used for DNA extraction. The qPCR reaction mix for one sample was as follows: 5 µL (of previously diluted to 5 ng/µL) genomic DNA was added to 15 µL reaction mixture, containing 10 µL LightCycler 480 SYBR Green I Master reagent (Roche Diagnostics GmbH, Mannheim, Germany), 2 µL of each forward and reverse primer, and 1 µL of PCR grade water.

The primers ZEA-F (5'-CTGAGAAATATCGCTACACTACCGAC-3') and ZEA-R (5'-CCCACTCAGGTTGATTTTCGTC-3'), targeting the zearalenone gene (Atoui et al., 2012), were used at a concentration of 2 pmol/μL. DNA amplification was performed in a 96 well block using a LightCycler® 96 Instrument (Roche Diagnostics GmbH). The PCR thermal cycling conditions reported by Atoui et al. (2012) were applied with slight modifications: 95°C initial incubation for 4 min followed by 40 cycles of denaturation at 95°C for 45 s, annealing at 60°C for 45 s, elongation at 72°C for 45 s with a final extension cycle at 72°C for 7 min. Quantification of the qPCR products was done using the LightCycler® software. Standard electrophoresis of the DNA samples was performed in an electrophoresis cell with 2% agarose gel (w/v) in 1x TAE buffer at 110 V for 45 min.

4.3.6 Mycotoxins analysis of kernels

A total of 41 mycotoxins (3-acetyl-deoxynivalenol, 15-acetyl-deoxynivalenol, aflatoxin B1, aflatoxin B2, aflatoxin G1, aflatoxin G2, agroclavin, alternariol, alternariol-methyl-ether, andrastin A, beauvericin, deoxynivalenol, deoxynivalenol-3-glucoside, diacetoxyscirpenol, enniatin A, enniatin A1, enniatin B, enniatin B1, fumonisin B1, fumonisin B2, fumonisin B3, fusarenon-X, gliotoxin, HT-2 toxin, mycophenolic acid, neosolaniol, nivalenol, ochratoxin A, ochratoxin alpha, ochratoxin B, patulin, roquefortine C, Roquefortine E, Sterigmatocistin, T-2 toxin, T-2 Triol, T-2-Glucoside, Tentoxin, Territrem B, Wortmannin and Zearalenone) were quantified in triplicate on treated and untreated malts. The analysis was carried out by UHPLC-MS/MS (Acquity™, Waters Corp., Milford, USA) according to DeColli et al. (2014).

4.3.7 Malt analysis and enzymatic activities

Malting loss was calculated based on the percentage weight loss from raw barley to the final malt (Briggs, 1998). Friability (degree of modification within a malt kernel) and extract content were analysed according to standard procedures (MEBAK Method 3.1.3.6.1 and 3.1.4.2.2, respectively) (MEBAK, 2011a). Total protein and β-glucan were measured according to standardised methods (MEBAK 3.1.4.5.1.1 and EBC 4.16.1). The enzymatic activities of the malts were assessed using the following assays: Ceralpha Method (Megazyme K-CERA 01/12) for α-amylase activity, Betamyl-3 Method (Megazyme K-BETA3 10/10) for β-amylase activity, and Azo-Barley Glucan Method

(Megazyme K-MBGL 03/11) for β-glucanase activity in malt and starter culture. To determine protease activity, an Azo-Casein Endoprotease assay was carried out, following the protocol described by Brijs et al. (2002).

4.3.8 Wort production and fermentation

Malts were ground using a laboratory disc mill (0.2 mm gap width, DLFU, Bühler GmbH, Germany). Wort was produced according to the standardised Congress Mash protocol (MEBAK 3.1.4.2) using an electronic mashing device (LB 8, Lochner Labor + Technik GmbH, Germany). Separation of wort from spent grains was done by filtration through folded filter paper of 7-12 µm pore size (Whatman 0860 ½, GE Healthcare UK Limited, UK). Gravimetic changes of the filtrate were recorded over time (0 - 90 min) to calculate filtration rate. A volume of 250 mL of filtered mash was then heated in round flasks and 150 mg pelletised hops (Target Type 90, Simply Hops, UK) were added at the beginning of a 60 min boil. The rest of the wort was used to measure viscosity using a falling ball viscosimeter (MEBAK 2.25.1) and the content of free amino nitrogen (MEBAK 3.1.4.5.5.1). The weight of the evaporated water was replaced with water after cooling. Boiled wort was tested for surviving LAB by spreading 100 µL of the chilled wort on MRS-Agar plates and incubating them at 30°C for 5 days. After a second filtration, the wort was collected in 500 mL conical flasks, inoculated with 240 mg dry yeast (Fermentis Saflager S-23, Lesaffre, France) and closed with an airlock. Fermentation was done at 15°C for 10 days, after which it was chilled to 2°C. After 28 days, the beers were filtered (Whatman 597 ½, Maidstone, UK) before further analyses were carried out. Throughout fermentation, extract levels were assessed after two and five days using a hand-held refractometer (Eclipse, Bellingham + Stanley Ltd, UK). Total yeast counts were assessed by swirling the flasks vigorously and taking 0.5 mL of sample at half height, centrifuging at 1900 x g for 5 min and re-dissolving the pellet in 2.0 mL Ringer's solution. The absorbance was then measured at 600 nm against pure Ringer's solution using a spectrophotometer (Helios Gamma UV-Vis Spectrophotometer; Thermo Electron Corporation, Basingstoke, England) and correlated to yeast cell counts done under a light microscope (VISIScope TL324P, VWR International, Darmstadt, Germany). After fermentation, alcohol content and apparent extract (Alcolyzer Beer ME, DMA 4500 M, Anton Paar GmbH, Graz, Austria) were assessed.

4.3.9 Statistical analyses

Minitab-software (Minitab Ltd., Coventry, UK, Version 17.0) was used for all statistical tests. One-way ANOVA was run for a level of significance set at $P < 0.05$. If F values were found significant, the statistical analysis was completed by Tukey's multiple comparison (HSD) test to further compare the groups to each other. Malting and brewing trials as well as all the analyses were carried out in triplicate and results are presented as means ± standard deviation.

4.4 Results and discussion

4.4.1 Microbiological analysis during malting

Culture-dependent analyses were chosen to study the living microbiota present on the surface of the kernels during malting. The high levels of LAB (up to 10.26 log cfu per grain in FW-T) detected in the treatments containing living *Lb. brevis* R2Δ starter cultures confirmed the successful application of the strain on the grain surface (Figure 6A). Because of the high number of LAB cells introduced with these malts, the choice of a suitable starter culture must consider aspects such as low hop-resistance and high heat-sensitivity in order to minimise the risks of cross-contamination in the brew-house. A slight increase in indigenous LAB was also observed for the control treatments (C-T and CA-T), confirming the natural growth of indigenous LAB cells. A significant reduction of the aerobic heterotrophic bacteria was observed for both FW-T and AFW-T, which showed a 96.9% and 99.0% cell reduction in the final malt, respectively (Figure 6B), compared to C-T. Application of LAB cells alone (LAB-T), however, showed no significant reduction compared to C-T, while the control containing added organic acids (CA-T) resulted in an average reduction of 76.2% in aerobic bacteria. These findings confirm that suppression of aerobic heterotrophic bacteria was caused by bacterial metabolites, e.g. organic acids, present in fermented wort, rather than from intercellular competition. Yeast levels increased throughout the first stages of the malting process (Figure 6C), but in this case growth was significantly promoted by the fermented treatments. Highest values were found for AFW-T before kilning (5.6 log cfu per grain), corresponding to ca. 5000 times the value for C-T. For both FW-T and CA-T, sharing the same acidity values, similar yeast stimulation was observed, whereas LAB-T had lower yeast levels. The observed increase in yeast population when lactic acid alone was added suggests the beneficial effect of acidification on their growth, which may have been due to a reduction in microbiological competition from acid-sensitive organisms. Symbiosis between LAB and yeasts are common in the food preparation and have been widely reported in other matrices, e.g. sourdough (Gobbetti et al., 1994), kefir (Shimizu et al., 1999) and dairy products (Viljoen, 2001). Laitila et al. (2007) found that a yeast starter culture applied to barley during malting positively contributed to the malt enzymatic activity (e.g. α-amylase, β-glucanase, cellulase and endo-xylanase) and several strains isolated from malt have shown antagonistic activity against moulds, including

Fusarium species. Boivin and Malanda (1997) showed that the yeast culture *Geotrichum candidum* could successfully inhibit *Fusarium* spp. and the associated mycotoxin deoxynivalenol (DON) could not be detected in the treated malt. In this regard, acidification could eventually promote beneficial synergistic effects when mixed starter cultures composed of LAB and yeast are added.

4.4.2 Impact on *Fusarium* infection

Since *Fusarium* contamination can lead to significant complications from both health- and quality-related perspective (Van Nierop and Rautenbach, 2006; Stübner et al., 2010), its early detection during the brewing process is of primary interest. The formation of red halos around kernels incubated on selective CZID-agar can identify the presence of *Fusarium* species. The halos increased in number during the germination step, with the largest amount of infected kernels corresponding to C-T and CA-T green malts (Table 10). The subsequent kilning generally decreased the infection level again to one or less infected kernels out of 15. Another method to estimate *Fusarium* infection on cereals, the count of red kernels, is widely used by maltsters to quickly assess the mycological status in malt batches (Geissinger et al., 2015; MEBAK, 2011a). In this study, C-T malt showed the highest values (31.0 ± 3.0 red kernels in 200 g malt), while the treated malts resulted in a significant reduction ($P < 0.0001$), with AFW-T and FW-T showing a count of 3 ± 1 and 2 ± 1 red kernels, respectively.

In comparison to the plate assay, red discolouration of malt can be visible only when a considerable amount of fusaria mycelia is already present on the kernels. On the other hand, plating of kernels on agar substrate can lead to distorted results regarding the true degree of infection as it encourages fungal enrichment of an otherwise minor *Fusarium* presence. For these reasons, comparison of both assays is of limited value.

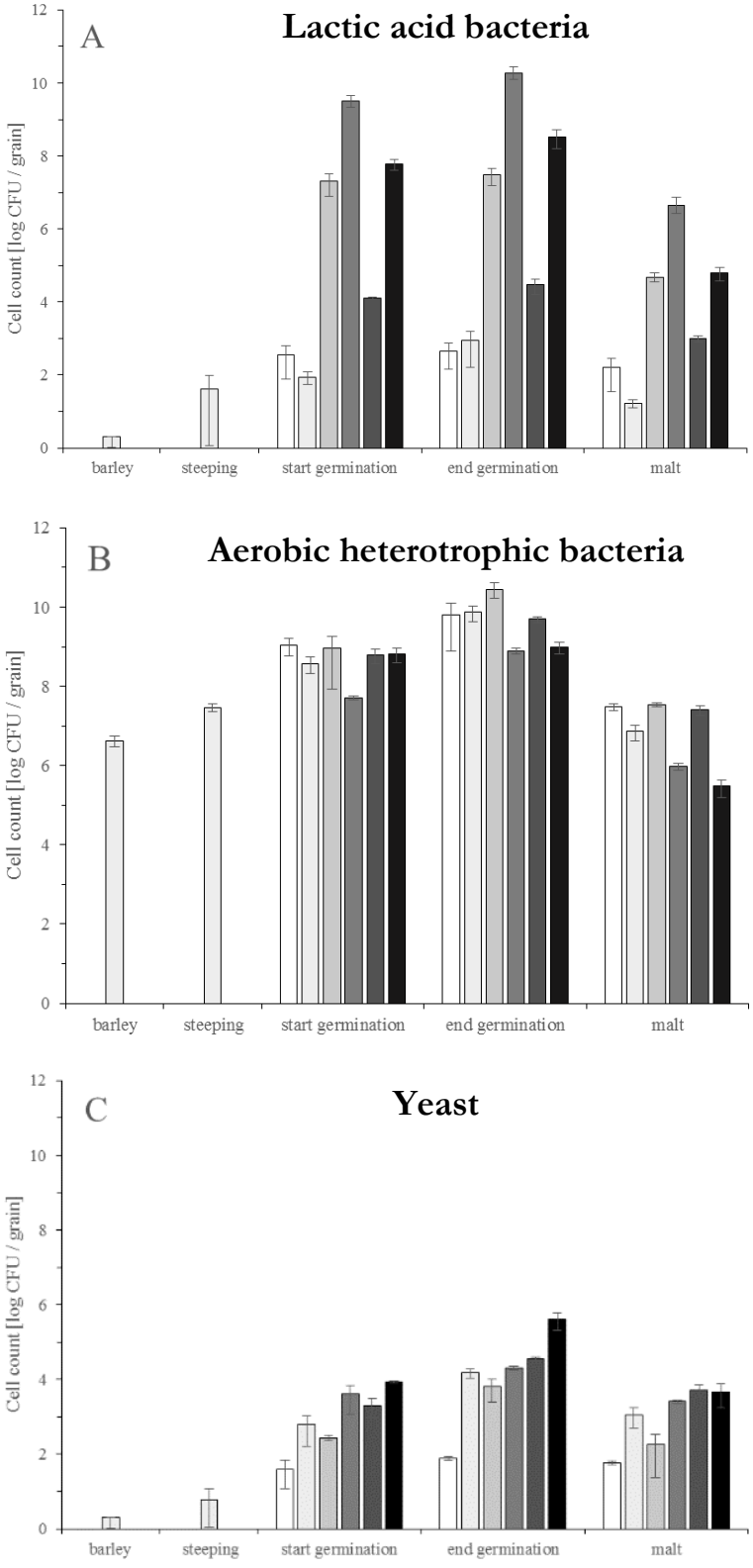

Figure 6. Growth of lactic acid bacteria (A), aerobic heterotrophic bacteria (B) and yeasts (C) throughout malting for the six treatments. In order from white to black: □ Control C-T; ■ Chemcial acidification CA-T; ■ Lactic acid bacteria LAB-T; ■ Fermented wort FW-T; ■ Pasteurised fermented wort PFW-T; ■ Acid fermented wort AFW-T.

Table 10. Infection by *Fusarium* spp. of barley, green malt and kilned malt assessed by plating on selective CZID agar (15 kernels) and visual counting of red kernels (in 200 g of malt); qPCR results on treated and untreated malts; levels of mycotoxins in treated and untreated malts. Control C-T; Chemcial acidification CA-T; Lactic acid bacteria LAB-T; Fermented wort FW-T; Pasteurised fermented wort PFW-T; Acid fermented wort AFW-T.

Analysis	C-T	CA-T	LAB-T	FW-T	PFW-T	AFW-T
Red halos on CZID agar [-]						
- raw barley (0 d)	2 ± 1	2 ± 1	2 ± 1	2 ± 1	2 ± 1	2 ± 1
- green malt (5 d)	4 ± 1	6 ± 0	ND	3 ± 1	ND	2 ± 1
- green malt (8 d)	5 ± 1	10 ± 0	2 ± 1	2 ± 1	2 ± 1	2 ± 1
- kilned malt (8 d)	1 ± 1	1 ± 1	ND	1 ± 1	ND	1 ± 1
Red kernels [-]						
- kilned malt (8 d)	31 ± 3	10 ± 1	22 ± 5	2 ± 1	19 ± 1	3 ± 1
***Fusarium* DNA [ng/g][a]**	78 ± 13	<LOD	40 ± 4	<LOD	<LOD	<LOD
Mycotoxins [µg/kg][b]						
- Deoxynivalenol-3-Glucoside	19.2 ± 2.6	18.9 ± 1.8	18.2 ± 4.1	<LOD	<LOD	<LOD
- Enniatin A	<LOD	<LOD	<LOD	<LOD	24.8 ± 0.0	<LOD
- Enniatin A1	51.5 ± 2.1	<LOD	<LOD	74.5 ± 4.9	104.0 ± 29.4	<LOD
- Enniatin B	58.0 ± 8.9	<LOD	21.0 ± 1.6	70.9 ± 22.7	269.7 ± 55.9	<LOD
- Enniatin B1	62.3 ± 9.9	<LOD	<LOD	83.3 ± 26.1	198.3 ± 42.2	23.8 ± 2.6

[a] Only DNA products from both *F. graminearum* and *F. culmorum* were considered.
[b] LOD for mycotoxins are reported in the Appendix 3.
ND: not detectable.

PCR-primers can be targeted to specific microbial groups, thus making it possible to monitor the presence, succession and persistence of certain microbial populations

within a complex ecosystem. Quantitative PCR (qPCR) was done to assess the non-viable load of *F. culmorum* and *F. graminearum* in the final malts. DNA extraction and qPCR analysis of pure fungal cultures showed that amplification with primers targeting the zearalenone gene (ZEA-F/ZEA-R) generated a 192 bp PCR product (Figure 7A). A serial 10-fold dilution of *F. culmorum* extracted DNA ranging from 60 ng/µL to 6 pg/µL was used to calculate threshold cycle (Ct) values. A standard curve was obtained by plotting the Ct value versus the logarithm of the concentration of each DNA dilution. The linear correlation coefficient of the standard curve was $R^2 = 0.962$ (Figure 7B). Heat dissociation analysis was done along with the separation of the amplicons on an agarose gel to verify that the amplified PCR product corresponded to the target gene (results not shown). Dissociation of the PCR reactions consistently produced one single peak at around 81-82°C, corresponding to the dissociation of the ZEA amplicon. The sensitivity obtained by adding *Fusarium* mycelia to disinfected malt samples was 6 pg/µL total extracted DNA. Generally, because of the low sensitivity of the qPCR methodology used in this study, only C-T and LAB-T malt could be quantified using the Ct values, whereas the other samples were below the level of quantification. Only melting peaks analysis could identify the amplicons for all samples.

Direct comparison of qPCR results with the two other methods is only of limited value, as already discussed earlier. Nevertheless, the main trends showed that application of acidic substrate combined with living cultures of *Lb. brevis* R2Δ, such as FW-T and AFW-T, could help to better control *Fusarium* infection compared to the addition of only acids (CA-T) or pasteurised wort (PFW-T). On the other side, the mere presence of LAB cultures (LAB-T) did not significantly improve the bioprotection of the grains. A portion of the bacterial metabolites appeared also to have a heat-labile nature, e.g. proteinaceous compounds, as suggested by the low reduction achieved with the pasteurised wort treatment (PFW-T). In order to prevent loss of these compounds, microfiltration or centrifugation instead of pasteurisation could be a better way of preparing a cell-free solution while retaining important bioprotective properties.

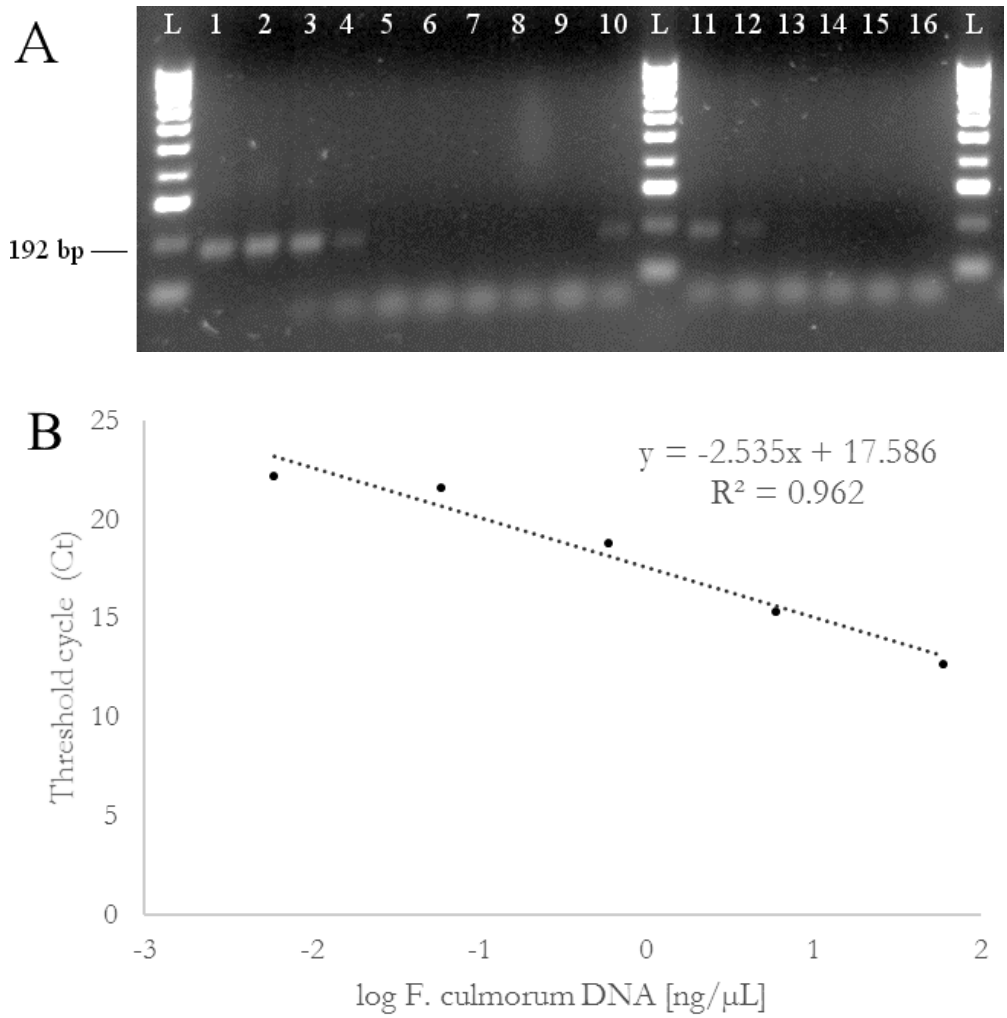

Figure 7. (A) Agarose gel electrophoresis of PCR products with ZEA-F/ZEA-R primers. Lane L, 1 kb HyperLadder™ (Bioline); Lane 1, *F. culmorum* TMW 4.2043 pure genomic DNA product at 60 ng/µL; Lane 2 – Lane 9, *F. culmorum* TMW 4.2043 pure genomic DNA product serially diluted 1:10; Lane 10, *F. graminearum* DSM 4528 DNA product at 60 ng/µL; Lane 11, C-T; Lane 12, LAB-T; Lane 13: CA-T; Lane 14, FW; Lane 15, PFW; Lane 16, AFW; (B) Standard curve of the ZEA assay used for quantification of *F. culmorum* and *F. graminearum* DNA. Cycle thresholds (Ct) were plotted against the DNA levels (ng/µL) of *F. culmorum* expressed on a logarithmic scale.

Overall, the most effective treatment was obtained with the acidic fermented wort (AFW), which contained less cells, but higher levels of organic acids and, thus presumably more bacterial metabolites. Comparison of treatments CA-T and C-T also indicated that organic acids partially inhibited the unwanted microbiota. However, the addition of mineral acids, e.g. lactic acid or phosphoric acids, can have acceptance limitations along the malting and brewing process, and they are forbidden in countries that produce beer in accordance with the strict German Purity Law (Kunze, 2010).

To conclude, an efficient substrate for bioprotection should aim for the careful balance between antifungal compounds and available wort nutrients. Introduction of nutrients with a partially unfermented substrate could potentially promote the growth of unwanted indigenous microorganisms, thus partially defeating the purpose of bioprotection.

4.4.3 Mycotoxin analysis

Mycotoxins are secondary metabolites produced by moulds as natural defence against environmental threats and several of them have been associated with human diseases (Lowe and Arendt, 2004). This is of concern for the brewer, as many of them can survive and accumulate during the brewing process. Oliveira et al. (2012) found that 78% of the mycotoxin deoxynivalenol accumulated during malting of barley was transferred into the final beer. The type and quantity of mycotoxins quantified on the malt kernels (Table 10) varied significantly between the different treatments. *Fusarium* mycotoxins of the enniatins (ENNs) family and deoxynivalenol-3-glucoside (D3G) were the only toxins detected in a quantifiable amount among the treatments. While FW-T malts contained no D3G toxin, the levels for the ENNs were raised, especially for PFW-T. A plausible explanation is that the left-over nutrients from the wort partially encouraged growth of the naturally present fungal population on the grains. Moreover, the low level of LAB cells in PFW-T could have led to less active competition between bacterial and fungal microflora. LAB strains have also shown an active role in detoxifying infected grains, either by affecting mycotoxin metabolism in fungi, or through adsorption by the bacterial cell wall structures (Dalié et al., 2010; Shetty and Jespersen, 2006). This was confirmed in this study, with lower levels of mycotoxins being found when LAB-T was applied compared to C-T. Franco et al. (2011) found that by using viable or dead LAB cells, they could reduce deoxynivalenol (DON) *in vitro* by 16-56% and 35-67%, respectively. In contrast, the treatment with the most acidic wort (AFW) significantly reduced the levels of D3G and ENNs compared to PFW-T. In this case, in addition to the high amount of cells, treatments containing a substantial amount of acid have been shown to be necessary to limit the initial fungal spread and the eventual mycotoxin production in the late stages of malting. This was confirmed by the better control of mycotoxin release during chemical acidification (CA-T) compared to C-T.

These results underline the importance of having both an acidic wort substrate, which can inhibit initial fungal spread, and high levels of LAB cells, which are important for mycotoxin reduction.

4.4.4 Malt, wort and beer quality

Malts were compared for relevant techno-functional and chemical quality characteristics and for overall fermentability, as reported in Table 11 and Table 12. Throughout the malting process, up to 10.5% of the barley original dry matter can be lost during steeping (0.5–1.5%) or through respiration (3.5–5%) and rootlet growth (2.5–4%). These processes contribute to what is known as malting loss. Microbial metabolites, such as organic acids, have been reported to interfere with grain metabolism and to slow down germination (46). In this study, reduction of rootlet growth (3–5 mm shorter) and delay of rootlet appearance was observed for all treatments containing acids, with the largest reduction (up to 30% less malting losses) registered for the treatment with the highest acidity (AFW-T) (Figure 8). CA-T and FW-T malts, both of which had the same acidity level, shared similar rootlet length values.

Nevertheless, hindered germination and decreasing malting losses can lead to suboptimal formation and/or activation of brewing-relevant enzymes during malting, which could result in a soluble extract of lower quality during mashing (Lynch, 1980; Pillane and Briggs, 1966). This was shown by Mauch et al. (2011a), who reported a reduction in losses for malted barley from 7.3 to 2.3% (w/w) by applying a *Lb. plantarum* culture in MRS broth. The authors were able to compensate for the overall lower enzymatic activity (α-amylase, limit dextrinase, and β-glucanase) by optimising the mashing profile (Mauch et al., 2011b).

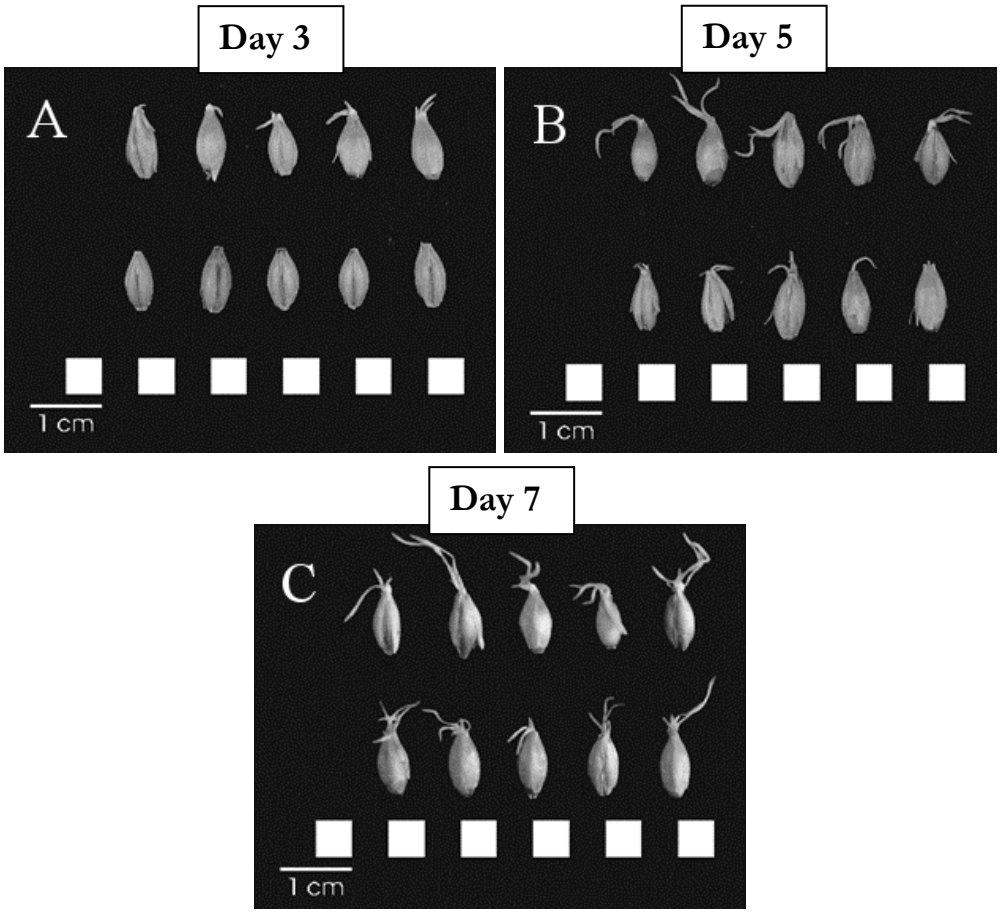

Figure 8. Rootlet growth of barley in control C-T (upper kernels) and acid fermented wort AFW-T (bottom kernels) on the (A) 3rd day, (B) 5th day and (C) 7th day of malting.

Table 11. Malt and wort quality attributes for the six treatments. Control C-T; Chemcial acidification CA-T; Lactic acid bacteria LAB-T; Fermented wort FW-T; Pasteurised fermented wort PFW-T; Acid fermented wort AFW-T.

Analysis	*Method*	*Unit*	C-T	CA-T	LAB-T	FW-T	PFW-T	AFW-T
Malt								
*Malting loss**	*MEBAK 3.1.3.2*	*%, d.m.*	10.81 ± 0.57 ^{a}	9.65 ± 0.92 ^{a}	10.74 ± 0.58 ^{a}	8.83 ± 0.97 ^{ab}	9.22 ± 0.99 ^{ab}	7.37 ± 0.23 ^{b}
Friability	*MEBAK 3.1.3.6.1*	*%*	90.8 ± 0.3 ^{a}	86.0 ± 0.2 ^{b}	84.9 ± 1.6 ^{bc}	85.5 ± 1.1 ^{bc}	87.3 ± 1.7 ^{bc}	88.8 ± 1.4 ^{ab}
a-amylase	*Ceralpha-method*	*Units/g*	290.0 ± 10.1 ^{a}	287.4 ± 8.1 ^{a}	278.5 ± 12.1 ^{a}	283.2 ± 9.3 ^{a}	298.9 ± 5.8 ^{a}	227.0 ± 18.2 ^{b}
β-amylase	*Betamyl-3 method*	*Units/g*	15.2 ± 0.6 ^{a}	15.9 ± 0.8 ^{a}	15.6 ± 0.4 ^{a}	15.0 ± 0.3 ^{a}	15.6 ± 0.6 ^{a}	14.9 ± 0.0 ^{a}
Endoprotease	*Azo-casein method*	*Relevant Absorbance*	1.45 ± 0.05 ^{a}	1.51 ± 0.02 ^{a}	1.51 ± 0.02 ^{a}	1.47 ± 0.02 ^{a}	1.52 ± 0.02 ^{a}	1.53 ± 0.01 ^{a}
β-glucanase	*Azo-barley glucan method*	*Units/g*	612.3 ± 59.8 ^{a}	597.4 ± 38.5 ^{a}	547.0 ± 16.3 ^{a}	517.0 ± 18.2 ^{a}	613.4 ± 39.1 ^{a}	542.4 ± 54.3 ^{a}
Total protein	*MEBAK 3.1.4.5.1.1*	*% (w/w), d.m.*	9.46 ± 0.16 ^{a}	9.60 ± 0.11 ^{a}	9.81 ± 0.30 ^{a}	9.65 ± 0.16 ^{a}	9.75 ± 0.13 ^{a}	9.84 ± 0.14 ^{a}
β-glucan	*EBC 4.16.1*	*% (w/w), d.m.*	0.47 ± 0.04 ^{a}	0.52 ± 0.05 ^{a}	0.59 ± 0.06 ^{a}	0.55 ± 0.07 ^{a}	0.51 ± 0.07 ^{a}	0.45 ± 0.04 ^{a}
Mash / wort								
pH mash	*MEBAK 3.1.4.2.7*	*-*	6.07 ± 0.02 ^{a}	5.90 ± 0.01 ^{c}	6.03 ± 0.04 ^{ab}	5.98 ± 0.01 ^{b}	5.86 ± 0.02 ^{c}	5.89 ± 0.01 ^{c}
pH wort	*pH-meter*	*-*	6.09 ± 0.02 ^{a}	6.05 ± 0.02 ^{ab}	6.08 ± 0.03 ^{a}	6.03 ± 0.02 ^{ab}	6.00 ± 0.04 ^{b}	6.04 ± 0.03 ^{ab}
App. extract	*MEBAK 3.1.4.2.2*	*%, d.m.*	85.0 ± 0.3 ^{a}	84.9 ± 0.1 ^{a}	84.2 ± 0.1 ^{c}	84.8 ± 0.2 ^{ab}	84.8 ± 0.1 ^{ab}	84.4 ± 0.1 ^{bc}

Continued

Analysis	*Method*	*Unit*	C-T	CA-T	LAB-T	FW-T	PFW-T	AFW-T
Filtration rate	*MEBAK 3.1.4.2.5*	*g/min*	12.2 ± 1.6 [a]	13.4 ± 1.0 [a]	12.7 ± 1.6 [a]	11.9 ± 1.8 [a]	11.7 ± 1.2 [a]	13.5 ± 1.6 [a]
Viscosity	*MEBAK 2.25.1*	*mPa.s*	1.569 ± 0.009 [a]	1.572 ± 0.006 [a]	1.579 ± 0.006 [a]	1.578 ± 0.009 [a]	1.578 ± 0.006 [a]	1.566 ± 0.004 [a]
FAN	*MEBAK 3.1.4.5.5.1*	*mg/L*	41.9 ± 2.8 [a]	44.2 ± 5.1 [a]	41.4 ± 2.0 [a]	44.9 ± 5.5 [a]	44.7 ± 1.6 [a]	42.7 ± 4.7 [a]

Each value was expressed as mean ± standard deviation analysed in triplicates. A different letter among values in a row denotes a significant difference at $P < 0.05$.

In earlier studies, LAB applied during malting have both improved as well as weakened the activities of malt enzymes (Laitila et al., 2006; Mauch et al., 2011b). In this study, α-amylase, β-amylase, endoproteases and β-glucanase in the malts showed similarities amongst all treatments, except for AFW-T, in which α-amylase activities were lower. It was previously shown by Mauch et al. (2011a) that the levels of hydrolytic enzymes, especially α-amylase and β-glucanase, were significantly reduced in treatments containing organic acids, suggesting interference with *de novo* synthesis of these enzymes in the aleurone layer.

Higher amounts of total protein in the final malts were observed for the treated samples compared to the untreated ones. This could be a consequence of the restricted germination of the kernels, with amino acids and peptides not be used for rootlet growth (Briggs, 1998). An increase in proteolytic activity in the presence of exogenously applied LAB during malting has been observed previously (Lowe et al., 2005b); however, in this study, application of LAB solutions did not affect the endo-protease activity. Although the strain had limited endogenous proteolytic activity, as demonstrated on milk agar plates (Appendix 1), this did not result in higher levels of free amino nitrogen in the final worts. In contrast, Mauch et al. (2011b) registered significantly lower values for total soluble and free amino nitrogen, indicating a limitation of proteolytic activity under acidic conditions.

Mashes from LAB-treated malts showed a marginal decrease in pH (up to 0.2 units lower for AFW-T). A pH of around 5.2–5.4 is associated with better enzymatic activity

during mashing, as many brewing-relevant enzymes have optimal activity within this range (Kunze, 2010). However, the lower pH values of the mashes produced using the treated malts (except for LAB-T) did not correlate with an improvement of their extract contents. Throughout mashing and mash filtration, the pH rose in all samples and the differences between the worts decreased (Table 12). This can be partially explained by the increasing release of buffering substances during the mashing process, e.g. phosphates and low-molecular nitrogenous compounds (Kunze, 2010). An increase in β-glucanase activity, which could have helped to lower β-glucan content and shorten lautering times, was not found in this study. Previously, the use of a *Lb. amylovorus* starter strain for biological acidification of a mash containing 20% unmalted and 80% malted barley, respectively, was found to reduce β-glucan levels when compared to a chemically acidified control. Although the starter culture tested negative for β-glucanase activity *in vitro*, the authors proposed that other, unidentified enzymes could have been released by the strain (Lowe et al., 2005b).

Throughout alcoholic fermentation of wort to beer, extract levels, yeast counts and final alcohol-by-volume (ABV) did not significantly vary between the different trials (Table 12). Premature yeast inactivation by LAB metabolites was not observed. The pH differences measured during mashing were not apparent during the brewing and fermentation processes, leading to no tartness in the final beers. Finally, worts were analysed for *Lb. brevis* R2Δ counts, but plating on MRS agar could not detect any viable LAB, thus excluding carry-over of a potential spoilage organism to subsequent brewing stages.

Table 12. Key attributes assessed during production and alcoholic fermentation. Control C-T; Chemcial acidification CA-T; Lactic acid bacteria LAB-T; Fermented wort FW-T; Pasteurised fermented wort PFW-T; Acid fermented wort AFW-T.

Analysis	*Method*	*Unit*	C-T	CA-T	LAB-T	FW-T	PFW-T	AFW-T
App. extract wort	*Density meter*	*% (w/w)*	9.17 ± 0.03 [a]	9.15 ± 0.01 [a]	9.11 ± 0.01 [a]	9.16 ± 0.02 [a]	9.16 ± 0.01 [a]	9.13 ± 0.01 [a]
App. extract beer	*Density meter*	*% (w/w)*	1.66 ± 0.03 [a]	1.66 ± 0.06 [a]	2.01 ± 0.48 [a]	1.80 ± 0.14 [a]	1.77 ± 0.09 [a]	1.81 ± 0.15 [a]
pH beer	*pH-meter*	-	4.30 ± 0.24 [a]	4.44 ± 0.26 [a]	4.03 ± 0.04 [a]	4.21 ± 0.46 [a]	4.08 ± 0.28 [a]	4.17 ± 0.41 [a]
Yeast count (48 h)	*Optical density*	*log cells/mL*	7.58 ± 0.05 [a]	7.57 ± 0.03 [a]	7.59 ± 0.03 [a]	7.61 ± 0.03 [a]	7.61 ± 0.01 [a]	7.59 ± 0.02 [a]
Yeast count (120 h)	*Optical density*	*log cells/mL*	7.71 ± 0.04 [a]	7.70 ± 0.05 [a]	7.72 ± 0.03 [a]	7.69 ± 0.02 [a]	7.69 ± 0.02 [a]	7.70 ± 0.06 [a]
ABV beer	*Alcolyzer*	*% (v/v)*	3.86 ± 0.08 [a]	3.81 ± 0.06 [a]	3.67 ± 0.03 [a]	3.79 ± 0.01 [a]	3.73 ± 0.16 [a]	3.68 ± 0.03 [a]

Each value was expressed as mean ± standard deviation analysed in triplicates. A different letter among values in a row denotes a significant difference at $P < 0.05$.

These results suggest that LAB application could improve malting and brewing productivity when considering the entire production chain from barley to beer. Because of rootlet growth inhibition with acid application and the concomitant lower malting losses, this resulted in an improvement of up to 3.1% extract yield compared to the control malt (C-T). In other words, the malting of 100 g raw barley (dry-matter) resulted in malt with 78.2 g soluble useable extract for AFW-T against 75.9 g for C-T. These extracts could be used to brew beer without apparent difference compared to the control.

Although the use of a malt-based medium as treatment solution could possibly lead to increased extract yield, no significant contribution could be detected between treated and untreated malts when leached extract was measured, thus attributing the increase in overall extract to the lower malting losses.

To increase the benefit from improved extract yields on an industrial scale, inexpensive carbon and nitrogen sources (e.g. spent wort, molasses), which contain the essential nutrients for LAB growth, could be potentially used as an alternative to malt extract (Endo and Dicks, 2014; Kotzamanidis et al., 2002). The utilisation of leftover malts that do not comply with regulations for good brewing malts (e.g. broken kernels) could be another approach to increasing the economy of malt houses. Finally, the re-use of the steeping-liquor is conceivable, as the presence of LAB coupled with high acidity should guarantee biological safety.

4.5 Conclusion

In summary, diluted wort fermented with *Lb. brevis* R2Δ imparted functionalities to this ingredient that enhanced microbiological and technological attributes of barley when added during malting. In detail, application of fermented wort promoted yeast population while decreasing aerobic bacteria and *Fusarium* levels, as well as improving extract yield, without apparent negative effects on the final wort and beer. These actions were primarily attributed to metabolites released by the strain into the spent wort medium, and secondarily to the LAB and their direct competition with the grain surface microbiota. In this regard, the most strongly acidified wort, containing viable lactic cells, appeared to advantageously contribute to both microbiological safety and production yield while retaining technological properties of malt.

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Chapter 5: Impact of buffering capacity on the acidification of wort by brewing-relevant lactic acid bacteria

Accepted as

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5.1 Abstract

Acidified wort produced biologically using lactic acid bacteria (LAB) has application during sour beer production and in breweries adhering to the German purity law (*Reinheitsgebot*). LAB cultures, however, suffer from end product inhibition and low pH, leading to inefficient LA yields. Three brewing-relevant LAB (*Pediococcus acidilactici* AB39, *Lactobacillus amylovorus* FST2.11, and *Lb. plantarum* FST1.7) were examined during batch fermentation of wort possessing increasing buffering capacities (BC). Bacterial growth was progressively impaired when exposed to higher LA concentrations, ceasing in the pH range of 2.9-3.4. The proteolytic rest (50°C) during mashing was found to be a major factor improving the BC of wort. Both a longer mashing profile and the addition of an external protease increased the BC (1.21 and 1.24, respectively) compared to a control wort (1.18), and a positive, linear correlation (R^2 = 0.957) between free amino nitrogen (FAN) and BC was established. Higher levels of BC led to significantly higher LA concentrations (up to +24%) after 48 h of fermentation, reaching a maximal value of 11.3 g/L. Even higher LA (max. 12.8 g/L) could be obtained when external buffers were added to wort, while depletion of micronutrient(s) (monosaccharides, amino acids and/or other unidentified compounds) was suggested as the cause of LAB growth cessation. Overall, a significant improvement in LA production during batch fermentation of wort is possible when BC is improved through mashing and/or inclusion of additives (protease and/or external buffers), with further potential for optimisation when strain-dependent nutritional requirements, e.g. sugar and amino acids, are considered.

5.2 Introduction

Lactic acid has a long history of use since its discovery in 1780 by the Swedish chemist Carl Wilhelm Scheele, who isolated it from sour milk and gave it the name "Mjölksyra", *milk acid,* based on its origin (Benninga, 1990). Owing to its versatile application as acidulant, flavour enhancer, and preservative, lactic acid occupies an important position in food processing (Abdel-Rahman et al., 2013). Commercial manufacture is either by chemical synthesis or by biotechnological process using lactic acid bacteria (LAB), with the latter accounting for ca. 90% of the total lactic acid produced (Hofvendahl and Hahn-Hägerdahl, 2000). LAB are generally strictly fermentative microorganisms that convert carbohydrates principally to lactic acid. Homofermentative strains are the preferred candidates for industrial LA production, as they release almost exclusively lactic acid from glucose (theoretical yields of >90%), while heterofermentative strains ferment glucose to lactic acid, but with ethanol/acetic acid and carbon dioxide as additional by-products (Endo and Dicks, 2014). LAB are characterised by good acid tolerance, however, nutritional requirements are high, owing to their limited ability to synthesize their own growth factors (refer to Table 2) (Axelsson, 1998; Van Niel and Hahn-Hägerdahl, 1999). Wort from malted barley is an inexpensive, natural substrate rich in fermentable sugars, free amino nitrogen (FAN), nucleic acid derivatives, vitamins and minerals, thus seemingly well suited to meet the high nutritional requirements of LAB (Bokulich and Bamforth, 2013; Charalampopoulos et al., 2002).

The benefits of lactic-fermented wort in brewing were first discovered in the early 1900s. Formerly regarded as beer spoiling bacteria, some LAB strains were selected to improve brewhouse yields and beer quality through the addition of lactic acid fermented wort to mash or wort (Henneberg, 1905; Jorgensen, 1909). Acidification of the mash is especially beneficial if adjuncts or malt of poor quality are being used, since it can compensate for the lower enzymatic activity (Lowe et al., 2004; Lowe et al., 2005). Additionally, some beer types like the "*Gose*", "*Berliner Weisse*", or the Belgian "*Lambics"* obtain their typical acid character through fermentation of indigenous LAB, and recently, especially among craft brewers worldwide, traditional and newly invented sour beer styles have received considerable interest.

During lactic fermentation, the decreasing pH represents a major inhibiting factor for LAB. In the case of pH-controlled batch fermentations, end-product inhibition is circumvented by trapping lactic acid as lactate salt with the addition of alkali agents (e.g. sodium hydroxide, potassium hydroxide, calcium carbonate). On a soluble starch

substrate, this can lead to higher LA production and yields (30–75.7 g/L LA, yields of 0.53–0.93 g LA/g utilised starch) when compared to a pH-uncontrolled batch fermentation (5.5–9.5 and 0.34–0.69, respectively) (Petrova et al., 2013). To recover the lactic acid, however, a subsequent downstream purification step is required (Abdel-Rahman et al., 2013). Another way to delay pH self-inhibition is to increase the buffering capacity (BC) of the substrate (Vriesekoop et al., 2012; Wang et al., 2015). In wort, nitrogenous compounds are regarded as major contributors to the BC (Coote and Kirsop, 1976; Taylor, 1990). Factors promoting the level of these compounds, such as total nitrogen content of malt and the extent of proteolysis occurring in mashing, will elevate the BC (Bamforth, 2001).

The objective of this study was to examine LA production using three brewing-relevant LAB species during batch fermentation of wort. The BC of the substrate was changed by adjusting the mashing profile, by adding a protease or by including buffers. An understanding of the inhibitory factors during lactic fermentation in wort can help brewers to control the process more accurately, with opportunities for improved resource efficiencies during production of new types of beers, e.g. sour beers.

5.3 Materials and methods

5.3.1 Bacterial cultures and reagents

For this study, three LAB strains (from 15 initial candidates; Appendix 5) were chosen based on preliminary trials. The strains *Pediococcus acidilactici* AB39, *Lactobacillus plantarum* FST1.7 and *Lb. amylovorus* FST2.11 were obtained from the culture collection of UCC (School of Food and Nutritional Sciences, University College Cork, Cork, Ireland) (
). The bacteria were maintained as frozen stock cultures at -80°C and regenerated on MRS agar by incubation at 30°C (FST1.7), 37°C (AB39) or 40°C (FST2.11) for 3–5 days. One colony forming unit was further inoculated in 10 ml of Congress wort (MEBAK 4.1.4.2) (MEBAK, 2011a) and incubated for 48 h at optimum temperature. The cultures were then stored at 4°C as stock solutions and sub-cultured in Congress wort at weekly intervals. All reagents used in the following trials were at least analytical grade from Sigma-Aldrich, St. Louis, unless stated otherwise.

Table 13. Bacterial strains used in this study.

Species	Strain	Metabolism	T_{opt}	Origin	Reference
Pd. acidilactici	AB39	Homofermentative	37°C	Teff sourdough	–
Lb. plantarum	FST1.7	Facultative heterofermentative	30°C	Malted barley	(1)
Lb. amylovorus	FST2.11	Homofermentative	40°C	Brewery environment	(2)

References in table: (1) Dal Bello et al., 2007; (2) Lynch et al., 2014.

5.3.2 Wort preparation

Barley malt (Pilsner Malt, Weyermann, Bamberg, Germany) was used for the preparation of wort. Milling was performed using a laboratory disc mill (DLFU disc mill, Bühler AG, Uzwil, Switzerland) with a gap size of 0.2 mm. Mashing was carried out in an electronic mashing device (LB 8, Lochner Labor + Technik GmbH, Berching, Germany) using a grist to liquor ratio of 1:7 (50 g in 350 ml brewing water). A control wort (CW) was obtained by performing mashing as specified for Congress mashing

(MEBAK, 2011b) (Figure 9). Further, an optimised wort (OW) was produced by extending the proteolytic rest (90 min at 50°C) and performing two separate amylolytic rests (30 min each at 62°C and 72°C, respectively).

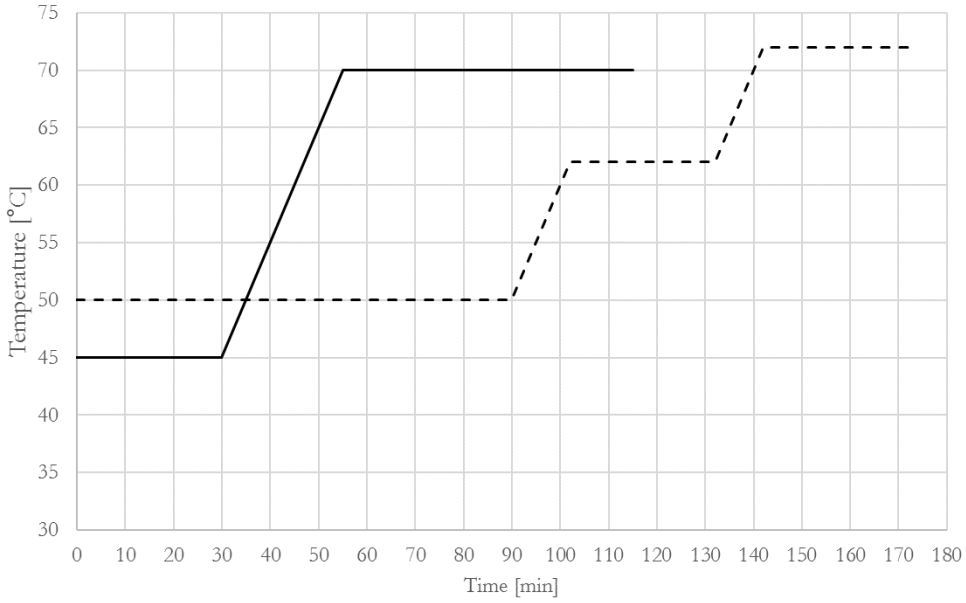

Figure 9. Mashing profiles for the preparation of control wort (CW) (black line) and optimised wort (OW) (dashed line).

Heating rate was 1°C/min and stirring speed was set at 100 rpm. Mashes were cooled to 25°C and filtered through folded filter papers. The filtrates were boiled vigorously for 5 min in round-bottom flasks and filled into containers under sterile conditions. Mashing trials were performed in triplicate.

The commercial protease Bioprotease N100L (bacterial metalloprotease from *Bacillus subtilis*; T_{opt} 50-55°C, pH_{opt} 6.0, Kerry Bio-Science, Carrigaline, Ireland) was added before mashing at 0.3 g/L in order to degrade proteins into smaller peptides and amino acids. Throughout this study, Bioprotease N100L will be referred to as "protease", and the respective worts were labelled CW+P and OW+P.

Trials with external buffers were prepared by adding a citrate-based buffer at a concentration of 1.44 g/L citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$) and 12.7 g/L trisodium citrate dihydrate ($C_6H_5O_7Na_3 \cdot 2H_2O$). This buffer was chosen over a phosphate-based and citrate-phosphate-based buffers, as it showed the highest BC over the pH range relevant during lactic fermentation in wort (between pH 6.0 to 3.0).

5.3.3 pH and end product inhibition

Control wort (CW) was buffered to pH 5.90 by adding 0.71 g/L citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$) and 6.35 g/L trisodium citrate dihydrate ($C_6H_5O_7Na_3 \cdot 2H_2O$). A pH gradient from pH 5.90 to pH 2.90 in steps of 0.50 ± 0.01 was prepared using either 1 M HCl or 90% DL-lactic acid (LA). Concentrations of HCl and LA accounted for 0.33, 0.73, 1.17, 1.64, 2.08, 2.63 g/L and 1.08, 2.70, 4.59, 9.01, 18.74, 51.71 g/L in pH 5.4, 4.9, 4.4, 3.9, 3.4 and 2.9, respectively. Solutions were then pipetted into 96-well microtitre plates and inoculated with 1% (v/v) overnight bacterial solutions. Optical density (OD) was measured every hour at 620 nm during 72 hours (Multiscan FC, Thermo Scientific, Waltham, Massachusetts, USA). Results were expressed as relative growth of the strain (in percentage) by comparing OD_{620} values at post-logarithmic phase between pH-corrected and control (pH 5.9) substrate.

5.3.4 Determination of buffering capacity

Buffering capacity was determined by automated titration of 50 mL of worts using 0.1 M HCl from initial pH to pH 3.0, with a rate of acid addition of 1.2 mL/min at a constant temperature of 25°C (836 Titrando with 800 dosino, Metrohm AG, Herisau, Switzerland). A fast determination of BC was also used according to a modified version of the method described by He et al. (2016), in which 375 µL of 1 M HCl were added to 25 ml of wort. Results for BC were calculated as follows:

$$BC = \log\left(\frac{\text{addition of } H^+ \text{ into wort}}{\text{increase of } H^+ \text{ in wort}}\right) = \log\left(\frac{V_{acid} \times c_{acid}}{V_{fin} \times 10^{-pH_{fin}} - V_{in} \times 10^{-pH_{in}}}\right)$$

where V_{in} and V_{fin} are the initial and final volume of the sample, while pH_{in} and pH_{fin} the respective pH values (He et al., 2016; Taylor, 1990).

The latter method was used to obtain a fast measurement of the BC build-up during mashing while limiting further enzymatic degradation. To do this, samples were taken every 15 min during the protease rest (50°C) from the mashing beakers and rapidly cooled on ice before being filtered through folded filter papers (Whatman 591 ½, GE Healthcare GmbH, Munich, Germany) and measured.

5.3.5 Fermentation

An overnight (16 h) inoculum of the bacterial strains was done in sterile Congress wort from 1% (v/v) stock culture. After incubation, the cells were washed once and resuspended in Ringer's solution (10 min at 3800 x g) and added at ca. 7 log cfu/mL in the substrates. Fermentation was carried out under static conditions in 50 ml Sarstedt tubes at optimal temperature for each strain. Total viable counts were determined after 0, 24 and 48 h of fermentation on MRS agar for AB39 and FST1.7 and on Congress wort agar (15% w/v agar in Congress wort) for FST2.11. Plates were incubated under anaerobic conditions at optimal temperature for 48 h.

Single carbohydrate (glucose, fructose, maltose, soluble starch) utilisation studies were done using a synthetic substrate based on the MRS medium and containing, per liter, 10 g of casein peptone, 5 g of meat extract, 5 g of yeast extract, 1 ml of Tween 80, 2.6 g of $K_2HPO_4 \cdot 3H_2O$, 3 g of NH_4Cl, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of $MnSO_4 \cdot H_2O$, 4 g of KH_2PO_4 and 0.5 g of cysteine-HCl. This medium and the sugar solutions were autoclaved separately. The final pH was adjusted to 5.8 with 1 M HCl. Fermentation was performed as outlined above.

5.3.6 Determination of metabolites

Organic acids (lactate and acetate) and sugars (fructose, glucose, maltose, and maltotriose) were analysed using HPLC-based methods. Sample preparation, analysis and quantification of the metabolites were done according to Peyer et al. (2015) after 0, 24 and 48 h of fermentation. Analyses of Free Amino Nitrogen (FAN) (MEBAK 3.1.4.5.5.1) and Free Amino Acids (FAA) (MEBAK 2.6.4.1.2) were carried out according to standard MEBAK methods (MEBAK, 2011a). Total starch was determined by the amyloglucosidase/α-amylase method (AACC method 76-13, procedure (g)) using a commercial kit (Megazyme International, Wicklow, Ireland).

5.3.7 Statistical analyses

Mashes, fermentations and analyses were carried out in triplicate, unless stated otherwise. The data was statistically analysed using Minitab, Version 17.0 (Minitab, Ltd., Coventry, United Kingdom). One-way ANOVA was used to compare means between different treatments. For multiple comparisons, Tukey's post hoc test was used with

95% confidence intervals. The statistical significance value for both ANOVA and regression analysis was set at $P = 0.05$. Values are given as mean ± standard deviation.

5.4 Results

5.4.1 Lactic acid yield from single sugar sources

To establish the preference for single carbon sources and the overall conversion yield to lactic acid, a defined MRS medium was formulated containing individual sugars and was fermented with each strain (Table 14). AB39 consumed more fructose than glucose, but converted glucose more efficiently into LA than fructose (yield of 89% compared to 71%, respectively). Maltose was consumed to a lesser extent and LA yields were low (12%). High assimilation was seen for FST1.7, and sugar-to-LA conversion rates were very high for all sugars (78 to 95%). Very high yield values (> 90%) are likely due to the production of LA from sugar impurities in the medium and/or from sources other than carbohydrates, e.g. amino acids (Christensen et al., 1999). FST2.11 consumed maltose and glucose in higher quantity compared to fructose, however, yields from glucose were poorer (40%) than for AB39. Finally, FST2.11 was the only strain producing LA (10.19 ± 0.2 g/L) when incubated in 2% (w/w) soluble starch in MRS. The consumption of starch amounted to 5.07 ± 0.77 g/L, while the rest of the carbon was provided by dextrins and simpler sugars present in the medium.

Table 14. Sugar consumption (%) and lactic acid yield $Y_{LA/S}$ (g LA/ g sugar) after 48 h of fermentation in MRS medium containing either 10% (w/w) fructose (Fru-MRS), 10% (w/w) glucose (Glu-MRS), or 10% (w/w) maltose (Mal-MRS).

	Fru-MRS			Glu-MRS			Mal-MRS		
	Sugar consumed [%]	LA [g/L]	$Y_{LA/S}$ [-] *	Sugar consumed [%]	LA [g/L]	$Y_{LA/S}$ [-] *	Sugar consumed [%]	LA [g/L]	$Y_{LA/S}$ [-] *
AB39	19.2	13.65 ± 0.24	0.71 ± 0.03	14.5	12.85 ± 0.10	0.89 ± 0.04	6.5	0.77 ± 0.04	0.12 ± 0.07
FST1.7	16.9	13.22 ± 0.17	0.78 ± 0.05	18.2	17.36 ± 0.10	0.95 ± 0.03	18.6	17.75 ± 0.12	0.95 ± 0.02
FST2.11	8.2	4.95 ± 0.16	0.60 ± 0.02	15.3	6.1 ± 0 .37	0.40 ± 0.01	15.8	10.36 ± 0.14	0.66 ± 0.05

*LA yield was calculated as a percentage from the amount (in g) of LA produced divided by the amount (in g) of sugar consumed.

5.4.2 pH inhibition of LAB

To estimate the effect of end-product accumulation on cell self-inhibition, control wort was pH-adjusted by adding either LA or HCl (Figure 10). Growth gradually declined with decreasing pH values, and dropped abruptly at pH 3.9 for FST1.7 and pH 3.4 for AB39 and FST2.11. The pH adjusted with LA caused a stronger inhibitory effect on the bacterial growth than HCl at pH 4.9 and lower. Growth stopped at pH 3.4 for all strains when pH was corrected with LA. Among the strains, FST2.11 showed higher viability at lower pH values. As an example, in terms of relative growth, FST2.11 was inhibited by 63% when growing in a LA-acidified wort (pH 3.9 with LA), while it reduced by 81% and 94% for AB39 and FST1.7, respectively.

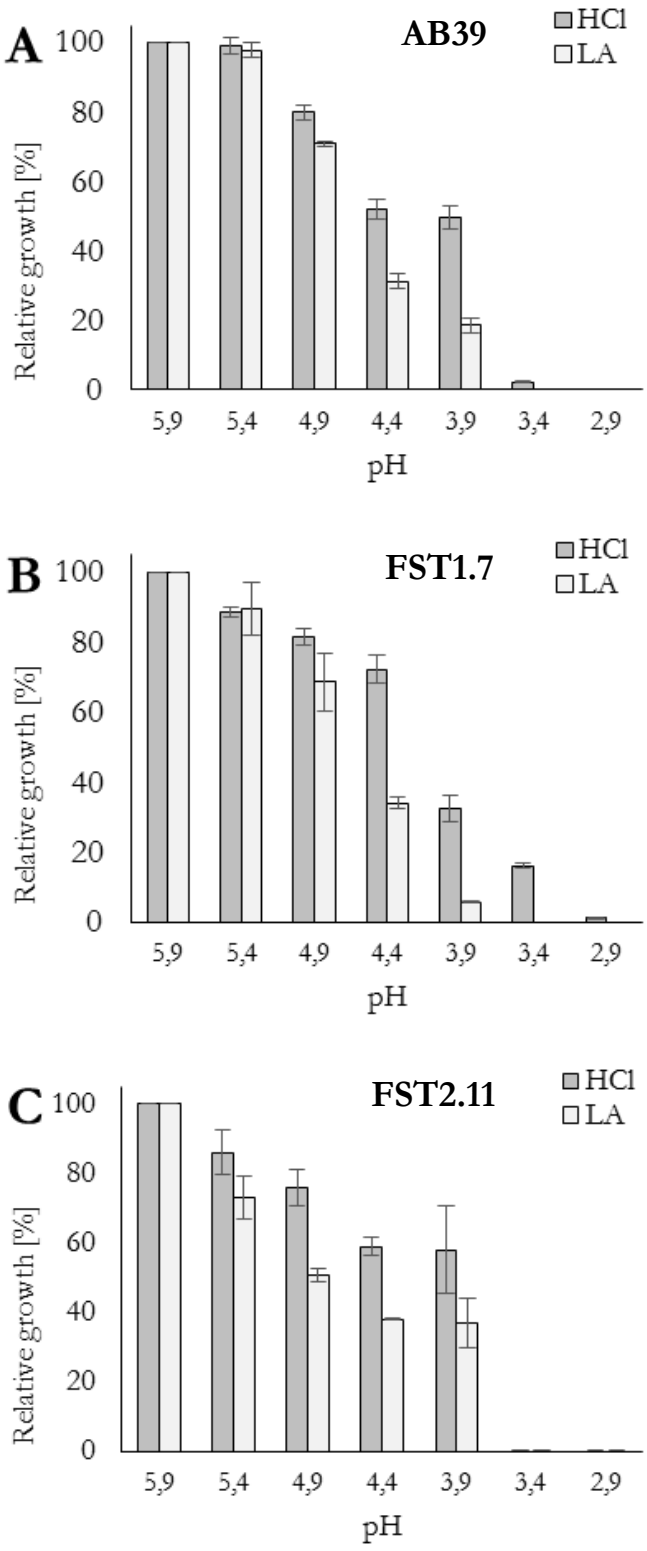

Figure 10. Relative growth (%) of the LAB strains calculated by comparing OD_{620} at post-logarithmic phase between pH-corrected CW (using HCl or LA) and control (pH 5.9). (A) AB39, (B) FST1.7, and (C) FST2.11.

5.4.3 Improvement of buffering capacity during mashing

Values of mash BC were quantified from mashing-in until the end of the proteolytic rest (50°C) (Figure 11). Starting at a value of 0.94, the BC rose to a maximum of 1.34 after 90 min (+43%) with no significant change over the last 15 min. A further increase in the BC during the remaining mashing process could not be observed.

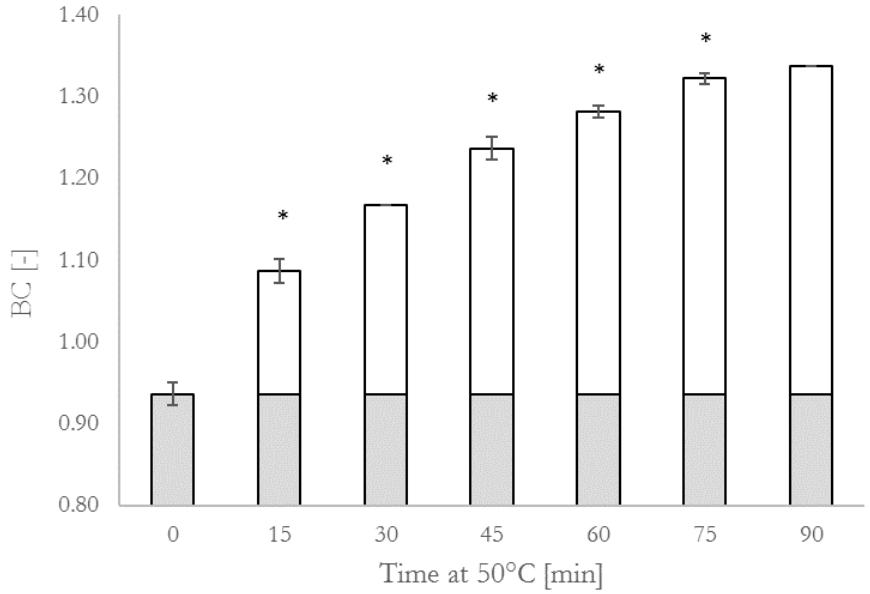

Figure 11. Increase in BC over time throughout the protease rest at 50°C during mashing. The superscript (*) indicates that BC value is significantly ($P < 0.05$) higher than the previous BC value.

To further improve the BC of both control wort (CW) and optimised wort (OW), an external protease was added at mashing in. The increase in FAN in the worts amounted to +23% in CW+P and +34% in OW+P. Regression analysis of corresponding BC and FAN showed that wort FAN had a statistically significant ($P = 0.000$) linear relationship with BC ($R^2 = 0.957$) (Figure 12).

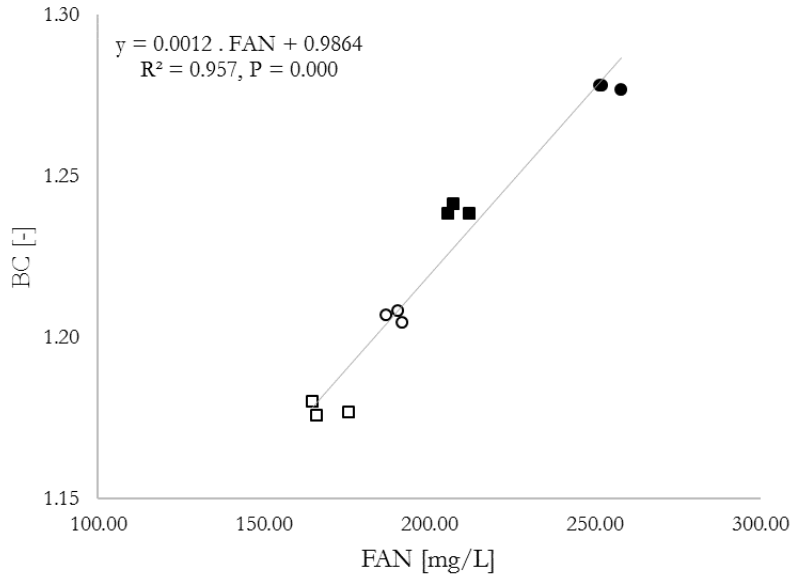

Figure 12. Correlation between FAN and BC with linear fitting. Contol wort (CW) (□), optimised wort (OW) (○), control wort with added protease (CW+P) (■), optimised wort with added protease (OW+P) (●).

5.4.4 Impact of buffering on LA production

The four worts were fermented with each strain over a period of 48 h (Figure 13). In addition, a citrate-based buffer was added to control wort (CW+B) and diluted (50:50 with water) control wort (CW0.5+B) to further improve LA production. Trials in diluted wort were performed to study the gradual deficiency of nutrients on LAB activity. The BC values of CW+B and CW0.5+B worts were 5.3 and 5.7 times higher than CW, respectively.

Compared with the control, the LA released in worts obtained by extending proteolysis and/or adding protease showed contrasting results amongst the strains tested. AB39 showed no notable increase in LA production, while FST1.7 achieved a significant ($P < 0.05$) increase of LA in OW+P compared to CW (8.65 ± 0.11 and 7.23 ± 0.29 g/L, respectively). This strain showed a linear correlation ($R^2 = 0.990$) between BC of the substrate and LA released. In contrast, FST2.11 reacted positively to the application of external protease, but only when added in the control wort (CW+P). The fermentation of this substrate led to high LA accumulation (11.3 g/L), corresponding to an increase of +24% compared to CW, while reaching the lowest pH values after fermentation (average of 0.25 lower than the other strains). The low final pH values reached during

the trials were likely to be responsible for cessation of bacterial growth. Fermentation of CW+B resulted in higher LA concentration for all strains (+32% to +53% compared to CW), with maximal LA released by FST2.11 (12.8 g/L). Lower LA values were found in the diluted, buffered wort (CW0.5+B). These were comparable to the values found for CW, but the significantly higher final pH values (4.25–4.71 compared to 3.05–3.31 in CW) suggested that, in this case, depletion of an essential nutrient(s) or co-factor(s) could have led to suboptimal fermentations.

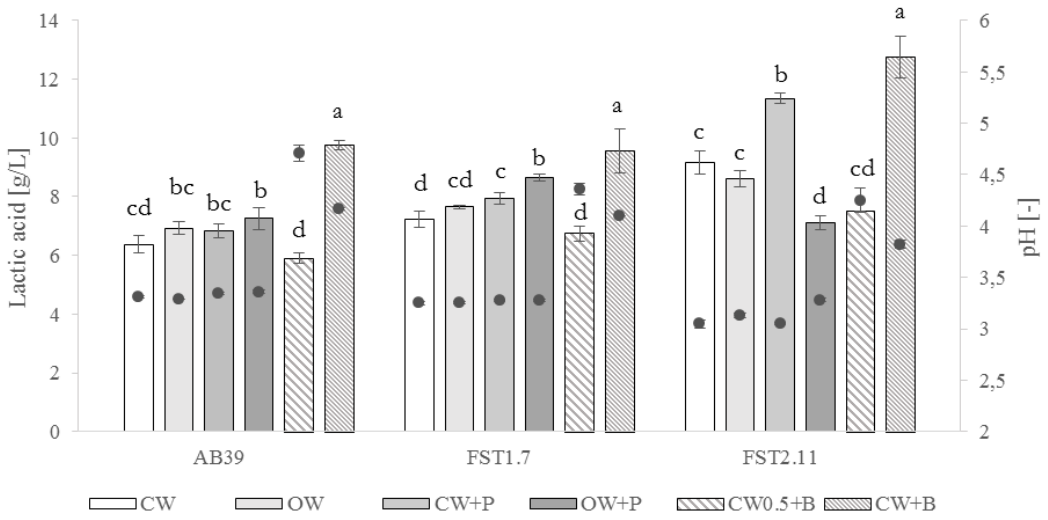

Figure 13. Lactic acid concentration (bars) and end pH (circles) of wort samples with progressively increasing BC after fermentation for 48 h. Contol wort (CW), optimised wort (OW), control wort with added protease (CW+P), optimised wort with added protease (OW+P), diluted buffered control wort (CW0.5+B) and buffered control wort (CW+B). Error bars indicate standard deviation. Statistical grouping of data was done according the post-hoc Tukey tests ($P < 0.05$) and indicated by superscript letters.

5.4.5 Metabolite consumption and cell count in buffered worts

A closer look at the sugar consumption (Table 15) revealed that fructose and glucose were the preferred carbon source of all strains, with complete assimilation of these monosaccharides in buffered trials, while residual sugars remained during control fermentation in CW for AB39 and FST1.7. The only culture that consumed maltose was FST2.11, while maltotriose was not utilised by any of the strains.

Table 15. Total viable cell count [log cfu/mL], sugar [g/L] and free amino nitrogen (FAN) [mg/L] levels after LAB fermentation (48 h) in control wort (CW), buffered control wort (CW+B) and diluted, buffered control wort (CW0.5+B).

	Cell count	**Fructose**	**Glucose**	**Maltose**	**FAN**
CW					
Unfermented	ND	1.89 ± 0.00	10.30 ± 0.14	67.72 ± 0.28	169 ± 6
AB39	8.67 ± 0.05	<LOD	8.25 ± 0.03	65.85 ± 0.25	159 ± 2
FST1.7	9.23 ± 0.13	<LOD	7.52 ± 0.21	65.02 ± 1.23	143 ± 1
FST2.11	8.18 ± 0.10	<LOD	<LOD	60.93 ± 0.28	144 ± 6
CW0.5+B					
Unfermented	ND	0.94 ± 0.06	4.99 ± 0.27	32.10 ± 0.26	78 ± 7
AB39	8.63 ± 0.08	<LOD	<LOD	34.17 ± 0.27	79 ± 1
FST1.7	8.95 ± 0.01	<LOD	<LOD	31.60 ± 0.16	57 ± 2
FST2.11	7.91 ± 0.10	<LOD	<LOD	29.27 ± 0.36	86 ± 6
CW+B					
Unfermented	ND	1.82 ± 0.03	9.78 ± 0.12	63.88 ± 0.88	165 ± 4
AB39	8.92 ± 0.06	<LOD	<LOD	65.79 ± 1.21	152 ± 3
FST1.7	9.47 ± 0.07	<LOD	<LOD	64.61 ± 0.43	126 ± 8
FST2.11	8.79 ± 0.09	<LOD	<LOD	59.37 ± 0.86	158 ± 2

LOD: limit of detection for fructose and glucose was 0.10 g/L and 0.15 g/L, respectively.
ND: not detectable (< 3 log cfu/mL).

Trials in CW+B led to the highest cell counts compared to CW and CW0.5+B, with the latter trials sharing similar values. Maximal cell growth for FST1.7 corresponded also to the largest decrease in FAN.

The analysis of 18 free amino acids (FAA) was performed in diluted buffered wort (CW0.5+B) to investigate substrate-specific causes for bacterial growth cessation. The results showed a strain-dependent consumption of single amino acids, with glutamine being completely assimilated by all strains, serine by AB39 and FST1.7, and arginine,

phenylalanine, tyrosine and tryptophan by AB39 and FST2.11. In this regard, FST1.7 depleted only two of the amino acids tested, while AB39 depleted up to six. The total consumption of FAA, however, correlated neither with FAN utilisation (Table 16), nor with the amount of LA produced.

Table 16. Amino acid [mg/L] concentration in diluted/buffered control wort (CW0.5+B) before and after LAB fermentation (48 h).

Amino acid	Control	AB39	FST1.7	FST2.11
Alanine	36.9 ± 1.5 ^{b}	82.3 ± 4.4 ^{a}	13.3 ± 3.2 ^{c}	75.9 ± 6.1 ^{a}
Arginine	47.6 ± 2.9 ^{a}	< 6 ^{b}	44.6 ± 2.9 ^{a}	< 6 ^{b}
Asparagine	32.0 ± 1.4 ^{a}	12.1 ± 0.8 ^{c}	15.1 ± 1.1 ^{b}	10.9 ± 0.3 ^{c}
Aspartic acid	27.5 ± 1.1 ^{b}	33.9 ± 1.7 ^{a}	10.6 ± 1.3 ^{c}	22.8 ± 3.5 ^{b}
Glutamic acid	22.2 ± 0.9 ^{c}	51.0 ± 2.9 ^{b}	9.1 ± 1.7 ^{d}	61.5 ± 5.3 ^{a}
Glutamine	41.5 ± 1.4 ^{a}	< 5 ^{b}	< 5 ^{b}	< 5 ^{b}
Glycine	11.3 ± 1.0 ^{b}	11.0 ± 0.9 ^{b}	4.9 ± 0.9 ^{c}	21.5 ± 1.6 ^{a}
Histidine	22.0 ± 1.7 ^{a}	26.5 ± 1.6 ^{a}	20.2 ± 1.2 ^{a}	21.1 ± 1.8 ^{a}
Isoleucine	23.4 ± 1.2 ^{a}	25.3 ± 1.2 ^{a}	8.9 ± 0.8 ^{b}	25.1 ± 1.7 ^{a}
Leucine	50.7 ± 3.0 ^{a}	37.6 ± 2.8 ^{b}	22.7 ± 2.5 ^{c}	40.8 ± 2.5 ^{b}
Lysine	30.2 ± 2.7 ^{a}	30.1 ± 3.0 ^{a}	13.3 ± 2.6 ^{b}	26.3 ± 7.3 ^{a}
Methionine	10.2 ± 0.3 ^{a}	< 10 ^{a}	< 10 ^{a}	< 10 ^{a}
Phenylalanine	41.7 ± 1.7 ^{a}	< 5 ^{c}	15.3 ± 2.8 ^{b}	< 5 ^{c}
Serine	23.6 ± 2.3 ^{a}	< 7 ^{b}	< 7 ^{b}	11.9 ± 1.1 ^{b}
Threonine	20.1 ± 1.0 ^{a}	10.6 ± 1.8 ^{b}	7.0 ± 0.6 ^{c}	13.6 ± 1.0 ^{b}
Tryptophan	14.0 ± 1.1 ^{a}	< 7 ^{b}	12.1 ± 1.1 ^{a}	< 7 ^{b}
Tyrosine	30.8 ± 1.6 ^{a}	< 6 ^{c}	11.9 ± 2.3 ^{b}	< 6 ^{c}
Valine	42.3 ± 1.4 ^{a}	44.9 ± 3.0 ^{a}	28.5 ± 3.6 ^{b}	45.3 ± 3.6 ^{a}
Total amino acids	518.5 ± 31.8 ^{a}	399.0 ± 36.1 ^{b}	271.4 ± 24.7 ^{c}	404.0 ± 38.1 ^{b}

$^{a\text{-}d}$ For each amino acid, a different letter in each row denotes a significant difference at $P < 0.05$.

5.5 Discussion

Batch fermentation of wort by LAB is a self-inhibiting process. Reasons for decreased acidification are the low pH reached within 24 to 36 h with the concomitant accumulation of weak acids. These can diffuse through the bacterial membrane in their undissociated form and release hydrogen ions (H^+) within the cytoplasm, impairing essential metabolic reactions and inhibiting critical cellular enzymes (Salmond et al., 1984). For LAB, this effect is particularly significant because of the high dissociation constant of LA (pK_a = 3.78) (Adachi et al., 1998). This explains the stronger inhibition effect that LA exerts on LAB growth compared to the strong acid HCl. In agreement with our results, Hongo et al. (1986) found that *Lb. delbrueckii* growth was substantially inhibited (> 50%) at values of 5 g/L LA and higher, concluding that the addition of lactic acid or lactates themselves had some adverse effect on cell growth rather than just lowering the pH.

To counteract the decreasing pH level during batch fermentation, the BC of the wort was improved, and the proteolytic rest during mashing was chosen as the main variable. A maximum BC (1.34) was reached after 75 min of mashing at 50°C, which decreased after a short wort boiling (1.21), likely due to the coagulation and precipitation of buffering high-molecular proteins and minerals as hot trub (Mathias et al., 2015). During sour brewing, this would be favourable for biological acidification performed directly after mashing-out (*mash souring*) instead of after wort boiling (*wort souring*). The temperature of the proteolytic rest corresponds to the activation of malt phosphatases (T_{opt} = 49-51°C), which release inorganic phosphorus mainly bound as phytic acid in grains (Lott et al., 2000). Both nitrogenous compounds (amino acids and peptides) and phosphates have long been considered as the most important buffering substances in wort (Coote and Kirsop, 1976; Hopkins and Kelly, 1929; MacKenzie and Kenny, 1965). However, a recent study by Li et al. (2016) questioned the relevant contribution of both free amino acids and phosphates to the overall BC. The authors concluded that because of the very low or very high pK_a values of the α-carboxylic acid group (range 1.7-2.2) or α-amino group (range 8.8-10.6), respectively, most amino acids contribute only poorly to the BC in the relevant pH range for lactic fermentation (Lundblad and MacDonald, 2010). Little contribution to the wort buffer system is made by aspartate (pK_a 3.86), glutamic acid (pK_a 4.25) and histidine (pK_a 6.04), which account for ca. 10% of the total BC at a wort pH of 5.5. The majority of BC is provided by peptides and polypeptides

containing these amino acids as well as organic acids (e.g. citrate) (Coote and Kirsop, 1976; Li et al., 2016, Taylor, 1990).

In this study, BC was progressively enhanced during mashing following this order: two-steps control mashing, three-steps mashing with long proteolytic rest, and mashing with addition of a protease. The latter method improved BC to a greater extent than by applying longer mashing profiles, resulting in a more time- and energy-efficient process. It was shown that higher FAN led to higher BC, however, the increase in LA production during fermentation was strain-dependent. The better pH resistance of FST2.11 enabled this strain to metabolise and sustain fermentation for longer. However, the similar final pH for each strain after fermentation as well as the residual glucose level (results not shown) suggested that the increase in BC in these substrates was not enough to avoid pH self-inhibition. Surprisingly, FST2.11 underperformed when inoculated in optimised worts (OW and OW+P). It can be speculated that due to the β-amylase rest applied in these mashing profiles, dextrins and other long-chained polysaccharides were degraded more extensively, affecting the metabolic activity of the amylolytic *Lb. amylovorus* strain. An alternative way to naturally promote BC in wort could be achieved by employing adjunct grains possessing higher mineral content / buffering capacity, e.g. quinoa, amaranth, teff or triticale (Arendt and Zannini, 2013). The flours of these grains, when included during sourdough fermentation, led to higher acidity values compared with a wheat control (Vogelmann et al., 2009; Wolter et al., 2014).

The addition of an external buffer into wort (CW+B) resulted in the highest LA concentrations and consumption of wort nutrients, while the pH values after 48 h fermentation were still high (3.82-4.17) for all strains. In contrast, fermentation of buffered, diluted wort (CW0.5+B) led to higher pH (4.25-4.71) and lower LA values compared to CW+B. In this case, the dilution of wort possibly caused an early depletion of essential micronutrient(s). On the other hand, LA concentrations in CW0.5+B and CW were similar, which supports the use of buffers while limiting the costs of malt ingredients. Although *Pd. acidilactici* AB39 showed a preference for monosaccharides over maltose when tested as single carbon sources, the strain failed to consume maltose in wort, and the limited acidification could be attributed to the lack of glucose and fructose. The generation of knowledge on the strain-dependent preference for the utilisation of carbon sources for the conversion into lactic acid allows the brewer to tailor the substrate preparation regime, e.g. malting, mashing or enzyme addition, to

improve the concentration of preferred sugars. As an example, glucose concentrations could be naturally increased by employing a special mashing procedure firstly introduced by Herrmann et al. (2003), which makes use of the endogenous maltase available in the malt to break down maltose and enrich more glucose in wort (Appendix 6). Different co-existing sugars in wort can be antagonistic for various sugar uptake mechanisms in bacterial cells, such as the suppression of the maltose transport system into the cell when glucose is present (Gänzle and Follador, 2012; Monedero et al., 2008). Furthermore, the initial fermentation of monosaccharides can reduce the pH to a level at which the maltose transport system is impaired (Guyot and Morlon-Guyot, 2001). This could be the reason why strain FST1.7, which fermented high amounts of maltose when present as single sugar, failed to do similar in the wort substrate. In contrast, FST2.11 was found to use maltose after glucose and fructose were depleted. For this strain, the remaining high concentration of maltose suggested that other factors than carbon source depletion were accountable for substrate limitations. For the reasons mentioned above, the screening of suitable strains for wort acidification should be performed directly in the original wort substrate, or in a synthetic medium containing wort sugars in the ratio found in wort, and ability to ferment maltose in the presence of monosaccharides present should be particularly examined.

LAB are generally auxotrophic for several amino acids (4 to 14 AA depending on the species) (Calderon et al., 2003), and possibly peptides (Van Niel and Hahn-Hägerdahl, 1999), as they are typically unable to synthetize them from inorganic nitrogen sources, e.g. ammonia. As a result, LAB depend on the organic nitrogen pool in the substrate or, if equipped with, on their proteolytic systems, e.g. extracellular proteinase and intracellular peptidases, to access essential AA and peptides from wort proteins (Chavan and Chavan, 2011). Bacterial proteolysis and/or *de novo* synthesis could explain the increase in alanine, aspartic acid, glutamic acid, and glycine found in this study. Metabolism of amino acids can also lead to by-products that can help the strain to cope with the acidic conditions of the substrate. Lactobacilli can convert glutamine, which was depleted by all strains in wort, into glutamic acid through a deamination reaction (Vermeulen et al., 2007), and levels of the latter were raised for strains AB39 and FST2.11 after fermentation. As mentioned above, the side chain of glutamic acid (pK_a = 4.25) increases the buffering capacity at pH values around 4.25, therefore, the conversion from glutamine to glutamic acid improves the buffering capacity of the growth substrate. This conversion was found to lead to higher biomass, to a greater

extent of acidification, as well as a higher final pH in sourdough systems (Vermeulen et al., 2007). As another preferred amino acid, arginine can be degraded by arginine deiminase to produce ATP and ammonia (NH_3) (Spano et al., 2004), with the latter being a strong base and helping to neutralise the acidic environment by forming ammonium ions (NH_4^+) (Fernández and Zúniga, 2006). Similarly, Liu et al. (2003) showed that serine deamination in *Lb. plantarum* may have a relevant role in strain survival during stationary phase by replenishing the pyruvate pool for energy formation and ammonia to counterbalance the acid environment. The complete exhaustion of several amino acids could explain the self-inhibition of the strains in this study. Moreover, deficiencies of certain amino acids could have negative consequences on the subsequent fermentation performance of brewing yeast, such as phenylalanine and tyrosine (Class 2 AA) and arginine (Class 3 AA) (Boulton and Quain, 2001).

As a means to counteract nutrient depletion, yeast extract has been the ingredient of choice when optimising LA production in synthetic media because of the supply of essential growth factors (nitrogenous compounds, vitamins B complex, minerals, buffering compounds) (Grant and Pramer, 1962). As shown by Wee et al. (2005), high levels of lactic acid (153.9 g/L) after batch fermentation with a *Lactobacillus* spp. isolate could be obtained on a substrate formulated exclusively with yeast extract, glucose, $(NH_4)_2HPO_4$ and $MnSO_4$. Nevertheless, exhaustion of a particular amino acid does not necessarily determine its essential nature. To this end, Loubiere et al. (1997) reported that the complete consumption of several amino acids after fermentation of a synthetic medium was not the cause for growth cessation of *Lact. lactis* subsp. *lactis*. When re-inoculated in a medium containing the remaining amino acids but devoid of the metabolic end-products, i.e. lactate, formate, acetate and ethanol, the strain regained normal growth rate. The authors concluded that inhibition was predominantly due to phenomena other than end-product inhibition and/or nutritional limitations, but associated with unidentified compounds produced during the fermentation.

5.6 Conclusion

Batch fermentation of wort by LAB is progressively inhibited due to the low pH and the accumulation of lactic acid. Increase of the BC through extended proteolysis and/or addition of protease was possible and, depending on the bacterial culture, this led to higher LA released. The further addition of external buffers significantly improved LA production, and shifted the cause for bacterial self-inhibition from low pH to nutritional deficiency. This was partially attributed to the depletion of assimilable sugar and/or essential amino acids. To further prolong acidification, optimisation of wort nutrient profile could be attempted by combining mashing regimes and ingredient addition following a strain-dependent approach.

5.7 Acknowledgments

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Chapter 6: Sour brewing: impact of *Lactobacillus amylovorus* FST2.11 on technological and quality attributes of acid beers

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6.1 Abstract

This study was conducted to compare the effect of different acidification methods using *Lactobacillus amylovorus* FST2.11 as a starter culture on the microbiological, technological and qualitative attributes of sour beers. Biological souring was performed in the mash after mashing, in the pre-boiling or in the post-boiling wort using the lactic acid bacterium *Lb. amylovorus* FST2.11. This strain was selected for its high sensitivity to hop, good growth at moderate levels of alcohol and fast acidification in wort. Alcoholic fermentation was carried out using a commercial *Saccharomyces cerevisiae* (Safale US-05) strain. Desired acidification of the unhopped substrates (ca. 5-6 g/L lactic acid) was achieved within 18 h of lactic fermentation. The lactic culture consumed maltose preferentially over monosaccharides, and uptake of free amino nitrogen was limited (8.0-12.6 mg/L). Yeast growth in soured substrates was delayed by 2 to 4 days compared to the unacidified control, but comparable end attenuations were achieved among all treatments after primary fermentation. Among the soured beers, lowest levels of off-flavours were found in pre-boil wort souring trials, while co-fermented beers led to opalescent beers containing high levels of total diacetyl and acetoin. The low pH and the high level of staling compounds such as acetaldehyde suggest that sour beers also suffered from low oxidative stability. Final beer qualities varied considerably depending on the souring method applied. Souring of wort by *Lb. amylovorus* FST2.11 before boiling was found to produce bright, tart beer with minor organoleptic failures while limiting the risk of bacterial cross-contamination within the brewing facility.

The renewed interest that sour brewing has received amongst brewing communities worldwide calls for a better understanding of the advantages and disadvantages of different souring methods. The results from this study could help brewers to choose the most suitable approach according to equipment capabilities and sensorial preference.

6.2 Introduction

Sour beers are recognised as one of the oldest commercial beer styles, and recently they have been rediscovered by brewers worldwide as an additional way to diversify their product portfolio (Bokulich et al., 2012; Tonsmeire, 2014). Different approaches can be chosen to produce sour beers. Traditionally, they can be obtained after prolonged acidification and ageing taking place over several months and involving different souring microorganisms, e.g. wild yeasts (e.g. *Saccharomyces*, *Brettanomyces*, and *Kloeckera* spp.), acetic acid bacteria (AAB) and/or lactic acid bacteria (LAB) (Annemüller et al., 2008; Spitaels et al., 2014). The synergies between the microorganisms will ultimately determine the complex flavour profiles of these products. Most notable examples of sour beers produced in this way are the Belgian *Lambics* and *Flanders red ales* and the German *Berliner Weisse*. On the other hand, increasing demand for more rapid production of acidic beers has called for alternative approaches that avoid the long fermentation and maturation times. Commonly, this involves the acidification of mash and/or wort before or during alcoholic fermentation. In this regard, the addition of food grade acid, e.g. lactic, phosphoric or citric acids, represents a straightforward way to acidify with evident benefits with respect to ease of dosage and consistency. However, beers with added refined acids were also described as lacking flavour complexity and their production is currently forbidden in facilities that oblige to the German beer purity law (*Reinheitsgebot)* (Narziss, 1984). Under these circumstances, the use of pure or mixed cultures of LAB has been preferred. Species such as *Lb. delbrueckii*, *Lb. amylovorus* and *Lb. amylolyticus* are commonly used as commercial starter cultures. These strains are capable of homofermentative metabolism of sugars, releasing almost exclusively lactic acid as the major organic acid (Endo and Dicks, 2014), although other secondary metabolites, such as diacetyl and acetoin, can be a determinant in the overall aroma and flavour profiles of cereal substrates (Blandino et al., 2003). Biological acidification by LAB commonly involves a separate batch acidification of wort, with partial addition to the main mash and/or wort in order to regulate the pH level)Kunze, 2010; Lowe et al., 2005a; Lowe et al., 2005b). During sour brewing, however, thorough acidification is needed to impart the characteristic sourness in the final beer. For this reason, acidification is often carried out using the whole brewing batch as substrate and only after the mashing process is completed in order to preserve enzymatic activity of the malt.

Since LAB can cause spoilage of conventionally produced beers, souring before boiling is preferable to reduce cross-contamination risks in the cold side of the brewing process. This can be done by either inoculating lactic cultures into the mash after mash out, or in the pre-boiling wort. In addition, this allows the brewer to use arbitrary hop regimes at levels, which could otherwise inhibit certain LAB cultures (Suzuki et al., 2006). Nevertheless, souring wort after the boiling step could be favored in order to retain desirable volatiles released during lactic fermentation, as well as to contain negative effects caused by boiling of acid wort (e.g. weak hop isomerisation and lower decomposition rate of dimethylsulfide (DMS) precursors) (Lewis, 1998). This latter method of acidification can be done before or as part of alcoholic fermentation.

To date, very little research has been conducted to understand the impact that LAB acidification methods cause on the processing and product quality of sour beers. This study aims to compare the fermentative profiles as well as the technological and final quality attributes of beers brewed using different souring practices.

6.3 Materials and methods

6.3.1 Strains and culture conditions

The strain *Lb. amylovorus* FST2.11, originally isolated from a brewing environment, was stored as frozen stocks in 40% glycerol at -80°C. The strain was routinely grown on Malt Extract (ME) agar plates (Fluka, Buchs, Switzerland) under microaerophilic conditions for 48 h at 40°C. Propagation prior to final inoculation was done overnight at 40°C in Congress wort (MEBAK I 3.1.4.9.1.2) (MEBAK, 2011a) made with commercial Pilsner malt (Weyermann, Bamberg, Germany). Alcoholic fermentation of acidified and non-acidified wort was carried out using a commercial, spray-dried *Saccharomyces cerevisiae* (Safale US-05, American Ale, Fermentis, Lesaffre, France) strain at a concentration of 0.1% (w/v) (ca. 7 log cfu/mL). All reagents used were analytical-grade from Sigma-Aldrich (Missouri, USA), unless otherwise stated.

6.3.2 Characterisation of LAB and yeast cultures

The amylolytic and proteolytic activities of *Lb. amylovorus* FST2.11 were tested qualitatively on starch and skim milk agar plates, respectively, according to the methods of Lowe et al. (2004) and Axel et al. (2015) (Appendix 1). Growth of *Lb. amylovorus* FST2.11 in hopped wort was assessed using a microtiter assay according to Haakensen et al. (2009) with modifications. Briefly, a range from 1 to 15 mg/L of isomerised hop extract (Isohop®, 30% (w/w) iso-α-acids, Barth-Haas Group, Nürnberg, Germany) was prepared in Congress wort (8.5% (w/w)). International Bitterness Units (IBUs) were calculated as 1 IBU ≈ 1 mg/L iso-α-acids. Similarly, alcohol sensitivity was assessed by diluting 96% (v/v) ethanol to a range between 1% and 10% (v/v). Growth under different extract levels was checked in a range between 2 and 20% (w/w) by producing a concentrated Congress wort using a higher initial grist-to-liquor ratio (50 g with 75 mL liquor). For all trials, microtiter wells were filled with 200 µL of mixed substrate and inoculated with ca. 7 log cfu/mL of an overnight FST2.11 culture. Absorbance was continuously recorded at OD_{620} over 72 h at 40°C. Likewise, acid sensitivity of yeast was assessed within a range of 1 to 15 g/L D/L-lactic acid in Congress wort (pH range of 4.4 to 2.9). After inoculation of ca. 7 log cfu/mL yeast cells in Congress wort, OD_{620} values were recorded over 72 h at 20°C. All trials were performed as biological triplicates.

6.3.3 Brewing trials

Commercial Pilsner malt was used as base malt for all beers. A pilot-scale (60 L) brewhouse comprising a combined mash-boiling vessel, a lauter tun, and a whirlpool tank was used for mash and wort production. Hopping was not performed during this study. The souring and control trials were performed as outlined in Figure 14. The beers were brewed in duplicate and each brew was, in turn, fermented (lactic and alcoholic fermentation) in duplicate.

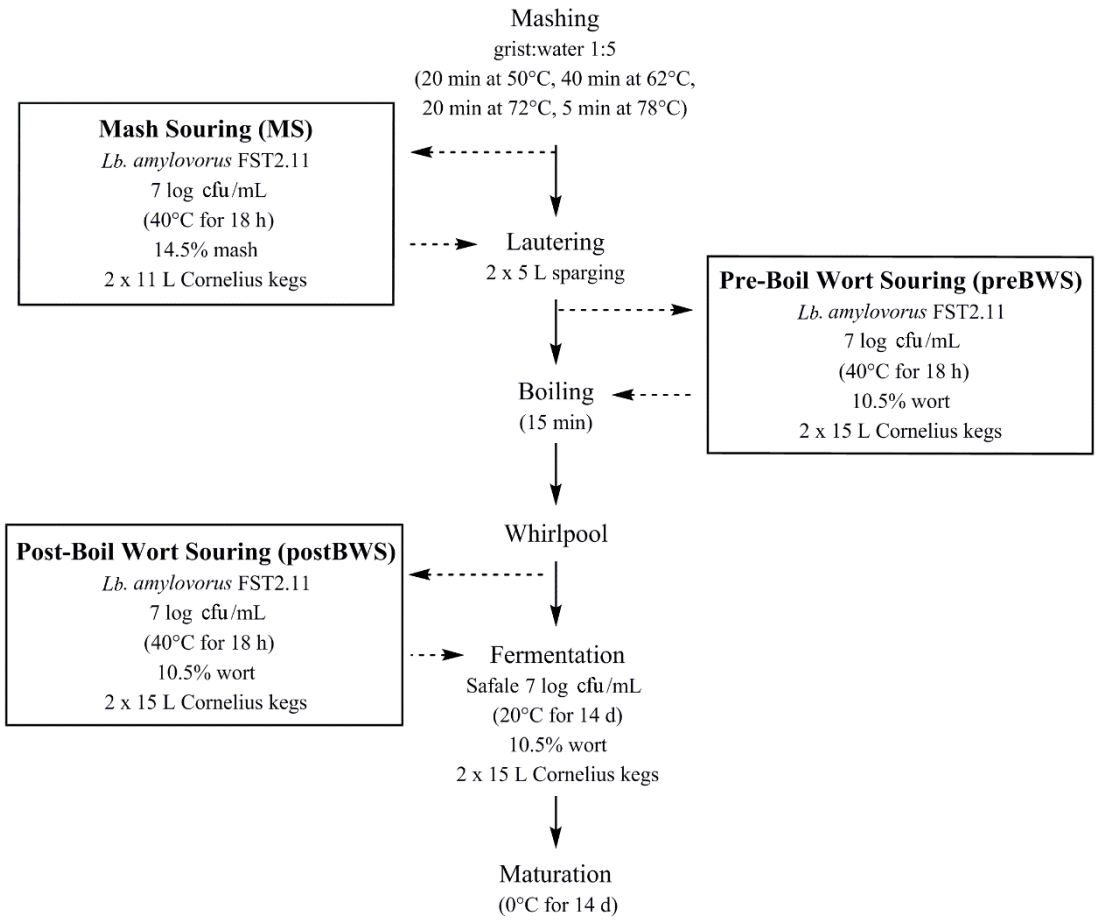

Figure 14. Schematic flowchart of the souring trials.

Yeast control (YC)

The malt was milled with a two-roller mill fitted with a 0.8 mm distance gap between the rollers. A grist-to-liquor ratio of 1:5 (6 kg grist with 30 L liquor) was chosen. A multi-step infusion mashing regime was employed as specified in Figure 14. The heating rate was at 1°C/min between the temperature rests. The mash was pumped in the lauter tun and two sparging steps of 5 L each were done. Lautering was performed at wort retrieval rate of 0.5 L/min after collecting 2 L of turbid wort for recirculation. The wort

was adjusted to an extract content of 10.5% (w/w) and boiled for 15 min. Hot trub precipitates were removed by means of a whirlpool, followed by cooling through a heat exchanger. In-line aeration was done using compressed clean air through a steamed aeration stone. The wort was filled in two Cornelius kegs (15 L each) and kept in a temperate room at 20°C for two weeks during alcoholic fermentation and maturation. Afterwards, the brews were racked in clean kegs and stored chilled at 0°C for another two weeks before bottling.

Mash souring (MS)

After mashing as described above, the mash was collected at mash-out temperature and filled in two kegs (11 L each). Carbon dioxide was flushed in the head space to provide micro-aerophilic conditions. The mash was cooled to a fermentation temperature of 40°C in around 2 ½ h. Propagation of FST2.11 was done initially in Congress wort (24 h at 40°C) and subsequently in fresh control wort (16 h at 40°C). An aliquot corresponding to 5% (v/v) (ca. 7 log cfu/mL) was inoculated in the mash followed by another CO_2 flushing. Fermentation was carried out for 18 h at 40°C until a pH of ca. 3.4 was reached. The two mashes were transferred back into the mash vessel, heated up to 78°C for 5 min and pumped to the lauter tun for lautering. The acidified wort was boiled and brewed as done for the control trials.

Pre-boil wort souring (preBWS)

After lautering and sparging, the collected wort was adjusted to an extract content of 10.5% (w/w) and held at 82°C for 10 min for sanitisation purposes. Subsequently, the wort was filled in two kegs (15 L each), flushed with CO_2 and cooled to 40°C for FST2.11 fermentation (18 h) as described above. The acidified wort was boiled and further brewed as performed for the control trials.

Post-boil wort souring (postBWS)

Boiled wort was collected in two kegs (15 L each) and lactic fermentation was done as described for the *preBWS* trials. After 18 h of LAB fermentation, wort was cooled to yeast fermentation temperature (20°C) and aerated with clean air for 1 min at a flow rate of 2.4 L/min through a sterilised aeration stone (0.45 μm pore size). Yeast fermentation and beer maturation were done as described for the control trials.

6.3.4 Microbiological analysis

Viable cell counts were determined throughout lactic fermentation (18 h), alcoholic fermentation (14 d) and in final beers (28 d). The colony forming units (cfu) of FST2.11 were determined using Malt Extract (ME) agar supplemented with 0.001% (w/v) cycloheximide to prevent yeast overgrowth. The plates were incubated anaerobically at 40°C and assessed after 48 h. To follow yeast growth, ME agar plates with added 0.01% (w/v) chloramphenicol to suppress bacterial growth were counted after aerobic incubation at room temperature for 3 days.

6.3.5 Chemical and technical analysis

The mashes were tested for the rate of filtration by gravimetric recording over 60 min through folded filter paper (Whatman 0860 ½, GE Healthcare UK Limited, UK) according to Lowe et al. (2005a). Viscosity of worts was assessed using a falling ball viscosimeter (MEBAK I 3.1.4.4.1) (MEBAK, 2011a) and determination of β-glucan was done following the McCleary method (K-BGLU 05/15, Megazyme International Ireland Ltd., Bray, Ireland). Mashes and worts were analysed for their buffering capacity according to He et al. (2016), extract (w/w) and alcohol content (v/v) (Alcolyzer Beer ME Analyzing System, Anton Paar GmbH, Graz, Austria), pH and total tritatable acidity (MEBAK II 3.2.3) (MEBAK, 2011b). Free amino nitrogen (FAN) in worts was determined using the Ninhydrin photometric method (MEBAK II 2.6.4.1.1) (MEBAK, 2011b). Sugars (fructose, glucose, maltose and maltotriose) and acids (lactic, acetic and butyric acid) in mashes, worts and beers were determined via HPLC (Agilent 1260 Infinity) using a refractive index detector (RID) or diode array detector (DAD) for sugar or acid analysis, respectively. Sample preparation and HPLC running conditions were performed as described by Peyer et al. (2015). The amount of L-lactic acid was assayed using a commercial enzymatic kit (K-LATE, Megazyme International, Kildare, Ireland). The final beers were analysed for the concentration of total soluble nitrogen (MEBAK II 2.6.1.1), total polyphenols contents (MEBAK II 2.16.1) (MEBAK, 2011b) and haze (Beer Haze meter, Haffman LTP-6B, Dr. Lange, Germany). Colour (MEBAK II 2.12.2) (MEBAK, 2011b) was measured after centrifugation of beer samples at 4500 x g for 5 min and foam stability was determined according to the sparging method described by Lomolino et al. (2015) with minor modifications. Briefly, 20 mL of decarbonated beer were placed at the bottom of a glass sintered tube (G-3 filter, pore size 16-40 μm, 3.2 ×

20 cm) and all the liquid was brought to foam by sparging carbon dioxide at a constant flow rate (0.35 L/min) and pressure (30 kPa) for 15 s. Liquid and foam volumes were recorded throughout 15 min of observation and foam stability was expressed as the half-time ($t_{1/2}$) of the foam height.

6.3.6 Fermentation by-products and sensory analysis

The following fermentation by-products were analysed using gas-chromatography-based methods: steam volatile aroma compounds (MEBAK II 2.23.6), highly volatile fermentation by-products (higher alcohols and esters) (MEBAK II 2.21.1), vicinal diketones (MEBAK II 2.21.5.1), acetoin (MEBAK II 2.21.5.4), bound sulfur dioxide (MEBAK II 2.21.8.2) and free dimethyl sulfide (MEBAK II 2.23.1.1) (MEBAK, 2011b). Sensory analysis was carried out by 10 panelists (MEBAK Sensory Analysis Method 3.2.1) (MEBAK, 2014) and involved the descriptive analysis (smell, taste, off-flavours) of the samples.

6.3.7 Statistical analyses

Minitab software (Version 17.0) was used for statistical calculations. One-way ANOVA was used to compare mean values between the samples. When F values were found to be significant, Tukey's multiple comparisons procedure was used to further determine any significant differences between the trials. The level of significance was determined at $P < 0.05$. Results are expressed as mean ± standard deviation.

6.4 Results and discussion

6.4.1 Characterisation of bacterial and yeast cultures

When selecting the right lactic culture for acidification during brewing, different strain-specific attributes should be considered such as adaptability to wort substrate, resistance to environmental stress conditions and spoilage potential of the culture. Above all, the LAB strain should not become a biological hazard in premises that produce and handle beer with low hop dosages. For this reason, hop-sensitive strains are preferred over hop-resistant ones for souring purposes. The growth of FST2.11 was negatively affected by hop acids and it was reduced by 22% and 66% when wort contained 1 and 2 IBUs, respectively, when compared to an unhopped substrate (Table 17). Iso-α-acids can cause sensitivity in LAB through dissipation of the transmembrane proton gradient, with subsequent decrease of the cytoplasmic pH and impairment of the metabolic activities of the cell (Simpson, 1993). Inhibition was further increased when the substrate was corrected to the average beer pH of 4.5 (80% and 79% inhibition for 1 and 2 IBUs, respectively), and at IBU values of 5, corresponding to the starting IBU for commercial beers, e.g. lagers, no growth could be detected. As a comparison, hop-resistant LAB species (e.g. *Pediococcus* spp.*, Lb. brevis*) have been found to grow in beer containing up to 29 IBUs after inoculation for 60 days (Geissler et al., 2016). Nevertheless, the use of a hop-resistant LAB strain as acidifying culture could be justified if souring is to be performed in an already hopped substrate.

Growth of FST2.11 was found to be affected at a concentration of 8% (v/v) and higher of ethanol. This result is in agreement with a study by Gold et al. (1992), who found that different *Lb. amylovorus* strains cultured on different carbohydrate sources showed good growth up to 8% (v/v) ethanol, with some strains being able to grow even up to 16%. Overall, the authors found that the majority of the tested *Lactobacillus* strains were able to grow at 4% (v/v) ethanol. This moderate alcohol tolerance of lactobacilli is an important asset to ensure continued acidification during mixed fermentations.

The growth of FST2.11 increased linearly with the wort extract content (R^2 = 0.996) until a level of 14% (w/w) extract. High-gravity wort can provide more nutrients as well as higher buffer capacity, which could promote LAB growth and delay self-inhibition due to low pH. Bacterial growth slowed at extract values above 16% (w/w), and reached a growth plateau between 18% and 20%. Similar to brewing yeasts, industrial LAB strains used in food and feed fermentations are likely to be exposed to osmotic stress

when high amount of sugars are present. Strategies by LAB to counteract hyperosmotic conditions involve the uptake or synthesis of compatible solutes within the cell and their release or degradation under hypo-osmotic conditions (Van de Guchte et al., 2002).
Growth of yeast linearly decreased (R^2 = 0.989) with increasing lactic acid concentration. Narendranath et al. (2001) reported that the minimal inhibitory concentration of lactic acid for *S. cerevisiae* was as high as 2.5% (w/v) when grown on a minimal medium (mineral salts, vitamins and glucose), while a concentration of 0.2-0.8% (w/v) began to affect growth rates and ethanol production. Rogers et al. (2016) reported that *S. cerevisiae* CBC-1 cultures failed to bottle carbonate sour beers with low pH (3.17) and total acidity of 2.5%. Although the yeast cells remained vital, a combination of low pH, high ethanol, lack of O_2, and other factors (e.g. limiting nutrients, acetic acid) were probably responsible for the metabolic alteration.

Table 17. Relative growth [%] of *Lb. amylovorus* FST2.11 and Safale US-05 yeast in wort substrates under different hop, alcohol, extract or acidity conditions.

Hop	**IBU 1**	**IBU 2**	**IBU 4**	**IBU 5**	**IBU 10**	**IBU 15**
FST2.11	78%	34%	17%	15%	9%	0%
Alcohol	**1% (v/v)**	**2% (v/v)**	**4% (v/v)**	**6% (v/v)**	**8% (v/v)**	**10% (v/v)**
FST2.11	97%	91%	89%	80%	75%	20%
Extract	**2% (w/w)**	**4% (w/w)**	**6% (w/w)**	**10% (w/w)**	**14% (w/w)**	**18% (w/w)**
FST2.11	12%	24%	39%	68%	88%	99%
Acidity (pH)	**1 g/L (4.4)**	**2 g/L (3.94)**	**4 g/L (3.51)**	**6 g/L (3.29)**	**8 g/L (3.17)**	**10 g/L (3.05)**
US-05	97%	93%	82%	73%	53%	43%

Absorbance values at post-exponential phase were considered, and percentages were calculated by comparing test and control values (set at 100%) corresponding to IBU 0 (pH 5.8), 0% (v/v) alcohol, 20% (w/w) extract and 0 g/L lactic acid (pH 5.88).

6.4.2 Microbiological growth

Microbiological composition was followed throughout lactic and alcoholic fermentation (LF and AF, respectively) (Figure 15). As mentioned above, the higher extract content of the mashes had a positive effect on the cell growth of FST2.11, reaching up to 8.5 log

cfu/mL, while values of up to 8.0 log cfu/mL were found in wort. This was partially attributed to the higher content of nitrogen compounds (amino acids, peptides, polypeptides) and phosphates that led to 28% higher buffer capacity in mash (2.36 ± 0.02) compared to wort (1.84 ± 0.02). Yeast growth was delayed by 2 to 4 days in the soured trials and counts increased to a lesser extent (up to 7.2 log cfu/mL) compared to the unacidified control, which in turn reached peak growth (7.7 log cfu/mL) within three days of alcoholic fermentation. This trend confirmed the pre-trial results (Table 17), which showed decreased growth of US-05 with increased substrate acidification. Although no significant changes were detected in the yeast population between *MS* and *preBWS* trials, yeast was negatively affected during *postBWS* fermentation. Here, the lactic culture continued to grow until the 3rd day of alcoholic fermentation, while yeast showed an initial decrease, but recovered after the 7th day. Bacterial population declined from the 5th day onwards, most probably due to increasing acidity, ethanol stress and substrate depletion. The growth of yeast cells could have been encouraged by nutrients, e.g. nitrogen compounds, released during autolysis of LAB. It is known that antagonistic and synergistic interactions between lactobacilli and yeasts and the exchange of their metabolites can induce significant differences in their growth and viability. Kedia et al. (2007) showed that co-fermentation of a 5% (w/v) malt suspension by yeast (isolate from "*Jiu Niang*") and *Lb. reuteri* mixed at different inoculation ratios (2:1 and 1:2) could enhance LAB growth in both cases, while weakening yeast growth. The authors suggested that the yeast supplied the bacterium with essential nutrients for growth, while being inhibited at the same time by increasing ethanol contents. Instead, studies done by Schönfeld (1938) showed that a yeast to LAB ratio of 4:1 (7 log and 2.6 log cfu/mL, respectively) during *Berliner Weisse* fermentation favored yeast growth within the first 18 hours of fermentation (7:1), then decreasing to 1.7:1 after 64 hours. Direct comparisons of these results with this study, however, are limited, since the yeast to LAB cell ratio at the start of alcoholic fermentation was in clear favor of the LAB cells (1:7.8, respectively).

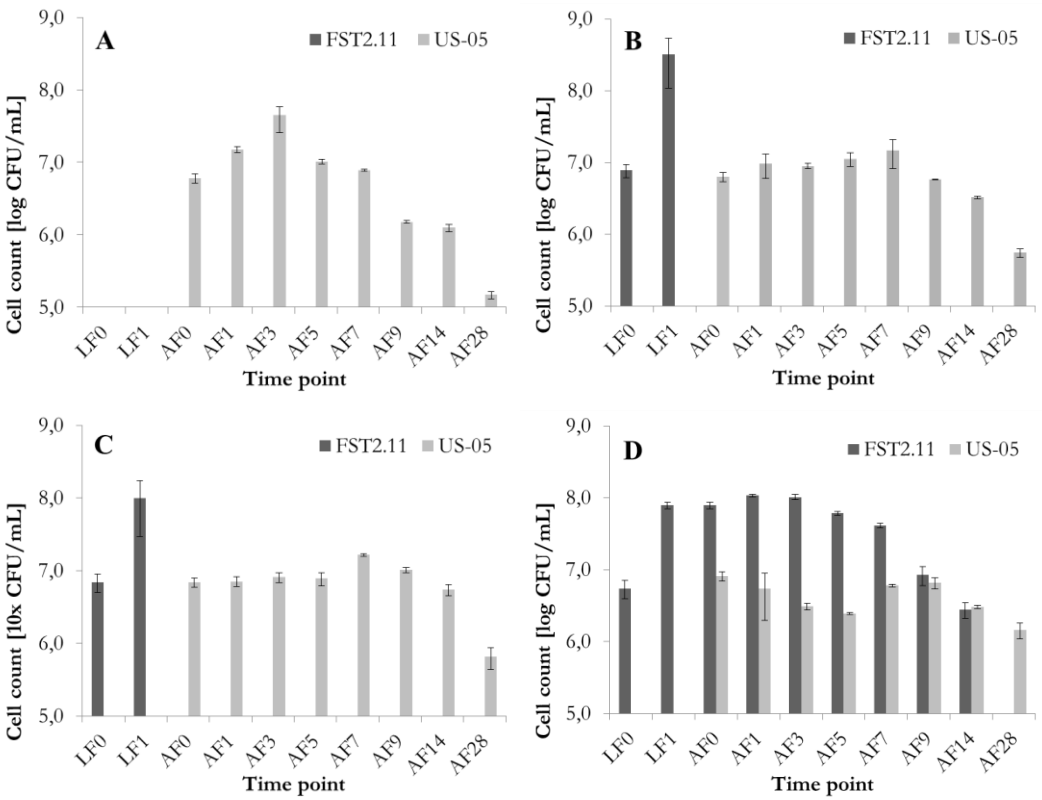

Figure 15. Cell counts of LAB and yeast cells throughout lactic (LF) and alcoholic (AF) fermentations in (A) Yeast Control (*YC*), (B) Mash Souring (*MS*), (C) Pre-Boil Wort Souring (*preBWS*), and (D) Post-Boil Wort Souring (*postBWS*).

6.4.3 Fermentation kinetics

Metabolite formation by LAB is a function of cell viability and vitality of the cell. The favorable growth condition during mash souring led to more extensive acidification (+56%) during *MS* compared to *preBWS* (Figure 16A). However, as shown above, FST2.11 slowed down growth above an extract content of 14% (w/w) (Table 17). For this reason, less acids were released proportionally to the extract content during mash fermentation (0.43 g/L lactic acid per unit extract) compared to wort fermentation (up to 0.51 g/L lactic acid per unit extract). Despite the increase in bacterial growth during the first three days of *postBWS* fermentation, additional acidification was contained, indicating limited LAB activity during alcoholic fermentation. Release of acid by-products (e.g. pyruvic, citric, lactic) as well as the assimilation and removal of buffering compounds (Bamforth, 2001) during the fermentative metabolism of yeasts caused further acidification in all trials and a final pH of 4.13 in the *YC* (Figure 16B). The pH level did not drop for the soured trials, with both *preBWS* and *postBWS* beers reaching lower pH (3.44 and 3.45, respectively) compared to *MS* beer (3.75). In addition, the *YC*

showed a faster reduction in the extract content compared to the soured trials (Figure 16C). This is in accordance with the higher yeast cell numbers recorded in this trial. Similarly, alcohol production was also faster in *YC*, reaching already after the 3rd day of alcoholic fermentation comparable levels of alcohol to that found only after the 5th day of fermentation of soured worts (Figure 16D).

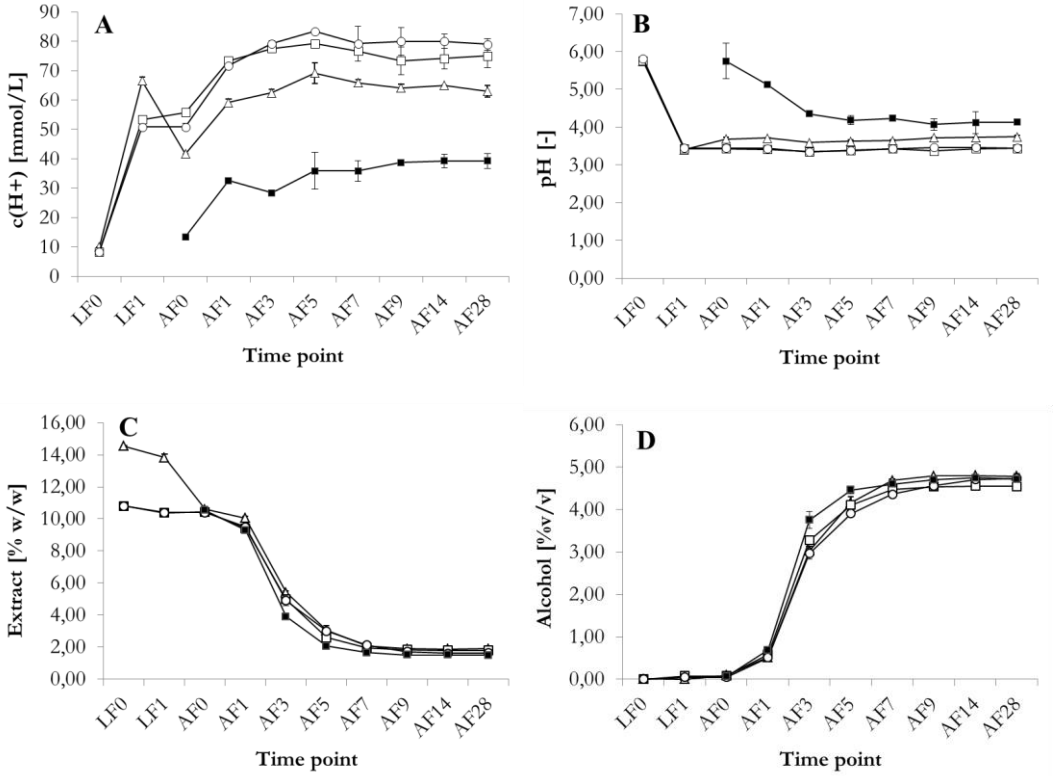

Figure 16. Total titratable acidity (A), pH levels (B), extract (C) and alcohol (D) in mash and/or wort throughout lactic (LF) and alcoholic (AF) fermentation for Yeast Control (*YC*) (■), Mash Souring (*MS*) (Δ), Pre-Boil Wort Souring (*preBWS*) (□), and Post-Boil Wort Souring (*PostBWS*) (○).

6.4.4 Metabolite profile

In accordance to the obligate homofermentative metabolism of *Lb. amylovorus* spp., which converts glucose almost exclusively into lactic acid (> 90% theoretical yield) (17), lactic acid was the main organic acid released by FST2.11 (Table 18). This was present in equimolar quantities (50.2-55.4% of L-lactate) of both enantiomers, and no acetic acid could be detected. The lactic acid concentration rose throughout alcoholic fermentation in all trials because of the fermentative yeast metabolism as well as evaporation losses during further brewing operations. The highest final concentration of lactic acid was found in *postBWS* beers at levels of 5.8 ± 0.1 g/L, with the majority (5.2 ± 0.7 g/L)

being release during lactic acid fermentation (Table 18). Maltose was quantitatively the preferred sugar consumed by FST2.11, followed by glucose and fructose, while maltotriose was not uptaken. Sucrose could not be metabolised by this strain. The yields of lactic acid accounted for 165% during *MS* and 92% and 103% during *preBWS* and *postBWS,* respectively, related to the sugar consumed and measured in this study. The very high yields during *MS* suggest that the strain produced lactic acid from other sources not quantified in this study, e.g. dextrins. *Lb. amylovorus* strains can express extracellular amylases (Zhang and Cheryan, 1991) that allow them to produce lactic acid directly from starch (63). *Lb. amylovorus* FST2.11 was found to possess amylolytic activities when tested on starch plates (Appendix 1). Therefore, large polysaccharides, which are partially retained during lautering, could have served as a carbon source for the direct production of lactic acid. Nonetheless, malt amylases still active during lactic fermentation could have increased sugar concentration before inactivation due to the low temperature and low pH.

Sugar consumption during alcoholic fermentation was faster in *YC*, with complete depletion of fermentable sugars after the 5th day. Yeast generally assimilates sugars in wort in the following order: sucrose, followed by the monosaccharides glucose and fructose, and finally maltose, and maltotriose (Hammond, 2002). In this regard, monosaccharides were quickly reduced after the first day of fermentation, maltotriose after the third day, and finally maltose after the 5th day. A similar priority of consumption was found also for the soured mash trials. Conversely, maltotriose and maltose were still present in both wort souring trials at the 5th day, but disappeared after the 7th day of fermentation. At the end of alcoholic fermentation, sugars were entirely consumed in all trials, and no significant changes in final extract content were detected between the trials, thus confirming complete extract attenuation in all beers (Figure 16C).

A limited consumption between 8.0 and 12.6 mg/L free amino nitrogen (FAN) was found during lactic fermentations, leaving sufficient FAN (116.7-131.2 mg/L) for adequate yeast fermentation (100-150 mg/L) (Briggs et al., 2004). Total consumption of FAN was higher during *YC*, pointing to a healthier fermentation during this trial, while lower consumption and higher end levels of FAN were registered for all the soured trials. A similar result was confirmed by Kedia et al. (2007), who reported reduced consumption of FAN during co-fermentation of *S. cerevisiae* and *Lb. reuteri* compared to the pure cultures. However, the proteolytic activities found for FST2.11 could have

increased the amount of free nitrogen during lactic fermentation and co-fermentation. This was suggested also by Rathore et al. (2012), who related the increase in FAN concentration observed at the end of *Lb. acidophilus* fermentation of different malt substrates to the action of proteases secreted by the bacteria. However, it cannot be excluded that nitrogenous compounds could have also been released as a consequence of LAB autolysis (Kedia et al., 2007; Oliveira et al., 2014).

Table 18. Sugars and free amino nitrogen (FAN) consumption and lactic acid production during lactic (LF) and alcoholic (AF) fermentation in Yeast Control (*YC*), Mash Souring (*MS*), Pre-Boil Wort Souring (*preBWS*), and Post-Boil Wort Souring (*PostBWS*).

Analysis	**Unit**	***YC***	***MS***		***PreBWS***		***PostBWS***	
		AF	LF	AF	LF	AF	LF	AF
Glucose	g/L	-8.4 ± 1.0	-0.5 ± 1.0	-9.2 ± 1.1	-1.7 ± 0.6	-7.6 ± 1.0	-0.8 ± 0.6	-6.8 ± 1.8
Fructose	g/L	-1.1 ± 0.1	-0.2 ± 0.5	-1.8 ± 0.6	-0.5 ± 0.4	-1.2 ± 0.2	-0.3 ± 0.3	-0.9 ± 0.1
Maltose	g/L	-56.3 ± 5.9	-2.9 ± 1.1	-58.0 ± 2.4	-3.5 ± 1.3	-53.9 ± 5.3	-3.8 ± 1.5	-54.9 ± 4.1
Maltrotriose	g/L	-16.2 ± 1.9	+0.1 ± 0.6	-17.4 ± 0.3	+0.3 ± 1.1	-16.1 ± 1.7	-0.0 ± 1.2	-17.6 ± 1.3
FAN	mg/L	-68.8 ± 5.2	-8.1 ± 4.2	-69.0 ± 5.4	-12.6 ± 3.9	-53.0 ± 7.8	-8.0 ± 4.3	-59.2 ± 10.1
Lactic acid	g/L	+0.3 ± 0.6	+6.2 ± 0.7	+0.4 ± 0.5	+5.4 ± 0.2	+0.2 ± 0.4	+5.2 ± 0.7	+0.6 ± 1.1

6.4.5 Brewing-relevant analyses

Characteristics such as filtration performance of the mashes were compared to the viscosities and β-glucan content of the resulting worts. The rate of filtration for the *MS* proceeded faster in the first 30 min, when compared to unacidified mashes (Table 19). Various factors could have played a role here, such as the decrease in extract during lactic fermentation, as well as the enzymatic degradation of macromolecules, e.g. starch, dextrins, proteins, that can positively affect the lautering rate (Webster, 1981). This was confirmed by measuring the viscosities of the clear worts obtained after filtration, which were lower for *MS* (1.61 ± 0.02 mPa.s) compared to the unacidified mashes (1.71 ± 0.02 mPa.s). Lowe et al. (2005a) ascribed improved lautering performances and lower viscosities of worts made from LAB-treated malt to the proteolytic activities of the starter cultures. However, a normalisation of filtration rates between soured mash and normal mash occurred after 30 min of lautering. This, on the other side, could be attributed to high number of suspended LAB cells (8.5 log cfu/mL) present in the

soured mash. The gradual accumulation of cells on the surface of the filter cake could increase the resistance to filtration (Tien and Ramarao, 2008), and ultimately slow down the wort run-off. The β-glucan levels in soured and unsoured mashes did not differ significantly, rejecting the degradation of β-glucans as a factor influencing faster initial filtrations (Lowe et al., 2004).

Table 19. Mash, wort and beer analyses of Yeast Control (*YC*), Mash Souring (*MS*), Pre-Boil Wort Souring (*preBWS*), and Post-Boil Wort Souring (*PostBWS*) samples.

Analysis	Unit	*YC*	*MS*	*PreBWS*	*PostBWS*
Mash					
Filtration rate					
5 min	mL	93.1 ± 0.5 ^{b}	114.4 ± 8.7 ^{a}	94.6 ± 3.8 ^{b}	97.2 ± 2.2 ^{ab}
15 min	mL	150.5 ± 6.3 ^{ab}	161.4 ± 1.2 ^{a}	151.6 ± 3.9 ^{ab}	150.7 ± 1.6 ^{b}
30 min	mL	176.7 ± 2.8 ^{a}	178.6 ± 4.7 ^{a}	177.3 ± 0.8 ^{a}	175.2 ± 0.7 ^{a}
60 min	mL	192.5 ± 4.4 ^{a}	186.9 ± 0.6 ^{a}	191.4 ±1.6 ^{a}	190.2 ± 2.2 ^{a}
Wort (10.5% (w/w))					
β-glucan	mg/L	373 ± 45 ^{a}	378 ± 21 ^{a}	391 ± 34 ^{a}	364 ± 18 ^{a}
Viscosity	mPa.s	1.74 ± 0.02 ^{a}	1.58 ± 0.02 ^{b}	1.64 ± 0.02 ^{b}	1.74 ± 0.02 ^{a}
Beer					
Haze	EBC formazin units	1.59 ± 0.24 ^{b}	0.43 ± 0.15 ^{c}	0.38 ± 0.04 ^{c}	3.38 ± 0.58 ^{a}
Total polyphenols	mg/L	106 ± 3 ^{a}	87 ± 5 ^{c}	90 ± 3 ^{bc}	102 ± 1 ^{ab}
TSN	mg/L	648 ± 28 ^{a}	611 ± 31 ^{a}	615 ± 15 ^{a}	650 ± 8 ^{a}
Colour	EBC units	6.4 ± 0.1 ^{a}	5.2 ± 0.0 ^{c}	4.9 ± 0.1 ^{c}	5.8 ± 0.0 ^{b}
Foam stability $t_{1/2}$	sec	189 ± 10 ^{ab}	224 ± 44 ^{a}	161 ± 14 ^{b}	206 ± 33 ^{ab}

Each value was expressed as mean ± standard deviation analysed as duplicate from two independent brews. For each attribute, a different letter in each row denotes a significant difference at $P < 0.05$.

Both *MS* and *preBWS* delivered clear beers after maturation (< 1 EBC formazin unit). Slightly more turbid was *YC*, and clear opalescence was found in *postBWS* beers. Compared to the control, the high turbidity in *postBWS* beers could be caused by the higher number of suspended yeast cells, which can create a persistent haze if they are not filtered out. Because both settled and suspended cells were considered for total cell counts in this study, no conclusion on the influence of acidification on the flocculation

extent of either microbial group can be drawn. However, differences in the flocculation ability of the yeast cultures can be expected, as this is strain-dependent and can highly depend on the environmental pH (Stratford, 1996). The pH present during boiling can affect the extent of protein precipitation, thus final haze. This takes place most efficiently at the isoelectric point (pI) of the individual proteins, with most of them having an pI of around pH 5.2 (Kunze, 2010). At lower and higher pH values, however, proteins gain positive and negative charge, respectively, and interactions are restricted. The low pH values of *MS* and *preBWS* suggested that protein interactions were not promoted during boiling. The values for total soluble nitrogen (TSN) in the final beers showed no significant difference among the trials. Nevertheless, total polyphenols, also involved in haze formation, were lower in both *MS* and *preBWS* beers compared to *postBWS* and *YC*, suggesting that an unidentified removal mechanism occurred during boiling under acidic conditions.

During the brewing process, Maillard and browning reactions can lead to darker beers through the formation of colour-bearing end-products. These temperature-dependent, non-enzymatic reactions occur faster at higher pH values (Schroeder et al., 1949). The colours of all soured beers were accordingly lighter, whereas the yeast control assumed darker colours. Pale beers were also reported in studies by Oliver-Daumen (1988) and Pittner and Back (1995) when biological acidification was applied during mash and wort compared to unacidified controls.

Together with turbidity, foam determines the first visual impression of beer quality to the consumer. Depending on the ability of the lactic cultures to degrade foam-active proteins, foam stability could be significantly influenced during sour beer production. However, this was seen in the past to not necessarily correlate with lower foam stabilities, as some of the proteins considered most important for foaming (LTP1 and proteins Z) are resistant to enzymatic digestion (Bamforth, 2012). Lowe et al. (2005) found that biological acidification by *Lb. amylovorus* FST 1.1 could improve the foam stability for a beer produced from 20% raw barley compared to that of 100% malt. The authors suggested that the proteolytic activity of the strain could have released more proteins to bind foam-destabilizing lipids. Even though *Lb. amylovorus* FST2.11 was found to possess proteolytic activities, foam analysis of soured beers showed that *MS* significantly promoted its stability compared to *preBWS*, but no differences were found compared to the *YC*. A correlation between nitrogen content and foam stability was not apparent in this case. However, a prolonged contact time between spent grains and wort

during *MS* could have led to the higher extraction of foam-stabilizing melanoidins from the malt husk (Jackson and Wainwright, 1978). Although these compounds have been suggested as not being as powerful as proteins in stabilizing foam, they are less susceptible to the action of proteases (Lusk et al., 1995). Melanoidins protect beers against the deleterious effects of lipid, as well as providing smaller bubbles (Bamforth, 1985). On the other side, the lysis of lactobacilli during boiling of *preBWS* could have released detrimental fatty acids affecting foam qualities. It is reported that stressed and unhealthy yeast cells can release, other than proteases, also lipids that can damage foam (Bamforth, 2012). However, lysates of yeast cells have been seen to also contain mannoproteins, which in turn can act as foam stabilizers (Blasco et al., 2011).

6.4.6 Flavour and sensory evaluation

Both yeast and lactic acid bacteria can add to the organoleptic profile by producing a variety of flavour compounds during fermentation (Lodolo et al., 2008). While yeast release mainly esters, higher alcohols, fatty acids, aldehydes, and sulfur compounds during alcoholic fermentation, LAB excrete primarily organic acids, aldehydes and few esters during fermentation of liquid cereal-based substrates (Peyer et al., 2016). Significantly higher levels of fermentation by-products, such as ethyl butanoate (tropical fruits), propan-1-ol (alcoholic) and 3-methylbutanoic acid (= isovaleric acid) (sweaty, foot) (Ara et al., 2006) were found in *postBWS* beers compared to the other trials (Table 20). The formation of volatile compounds in mixed fermentation is strongly influenced by the activity and vitality of LAB and the yeasts cultures and the release of metabolites that can act as flavour precursors. In this regard, amino acids likely released from the LAB proteolytic activity can be fed into the Ehrlich pathway of yeasts to form aldehydes, alcohols or carboxylic acids, e.g. isovaleric acid (Boulton and Quain, 2001). 3-mmethylbutanoic acid, also known as isovaleric acid, is a key odorant in different fermented foods, especially in cheese. It was reported by Guerzoni et al. (2007) that this compound was over-produced as a response to acid stress from both pure cultures of *Lb. sanfranciscensis* and *S. cerevisiae* as well as in mixed fermentation of a liquid sourdough. The same author found that acid stress increased the levels of medium chain fatty acids (FAs). FA levels were slightly raised for *postBWS* beers, but not significantly different from the other beers. Considerable amounts of total diacetyl and acetoin were found in *postBWS* samples. Diacetyl, a compound responsible for a butterscotch off-flavour, was above the sensory threshold for these beers, however, was not detected by the sensorial

panel. Diacetyl is formed through the non-enzymatic decarboxylation of α-acetolactate, a metabolite expelled by both LAB and yeast cultures during fermentation (García-Quintáns et al., 2008; Suomalainen and Ronkainen, 1968). This conversion is accelerated at lower pH values, as seen by Haukeli et al. (1978), who reported a 4-fold increase when the pH in fermentations was lowered from 5.5 to 4.0. After primary fermentation by yeasts, the diacetyl formed is normally re-absorbed by the yeast cells and reduced to acetoin (cream, butter), which, in turn, is converted to 2,3-butanediol. The results suggested that this conversion was not completed in the *PostBWS* soured beers. The substantial impairment of yeast metabolism during these trials could have caused a suboptimal re-absorption of diacetyl. Similarly, acetaldehyde (green apples, fruity) (Meilgaard, 1979) was detected over its sensory threshold only in the soured beers, with the highest level registered for the *MS* trial (40.25 mg/L), at four times higher than its sensory threshold in beer (10 mg/L), and eight times higher than the *YC*. This compound is formed during alcoholic fermentation as an intermediate metabolite and re-absorbed by the yeasts after primary fermentation to be converted to carbon dioxide and ethanol (Lodolo et al., 2008). Numerous causes have been identified that enhance acetaldehyde levels (Baert et al., 2012). On one side, unhealthy yeast cells present at the end of fermentation can be impaired in their ability to re-absorb or finish the conversion from acetaldehyde to ethanol. Additionally, exposure to oxygen can lead to the oxidation of ethanol back to acetaldehyde (Bradshaw et al., 2011). It is known that oxidative stability of beer is reduced at low pH values, contributing to the formation of staling compounds (Grigsby et al., 1974). The lower amount of polyphenols in the soured beers could have also led to higher oxidation events, which are triggered by the accelerated formation of reactive oxygen species (Kaneda et al., 1997). Acetaldehyde is particularly undesired, as it can be further involved in reactions leading to other staling compounds (Vanderhaegen et al., 2006).

Table 20. Fermentation by-products [mg/L] analysed in of Yeast Control (*YC*), Mash Souring (*MS*), Pre-Boil Wort Souring (*preBWS*), and Post-Boil Wort Souring (*PostBWS*) beer samples after a total of four weeks of fermentation and maturation.

Compound	*YC*	*MS*	*PreBWS*	*PostBWS*	Sensory threshold*
Esters					
Ethyl butanoate	0.22 ± 0.03 ^{b}	0.15 ± 0.01 ^{b}	0.18 ± 0.01 ^{b}	0.33 ± 0.03 ^{a}	0.4 (47)
2-Methylpropyl acetate	0.05 ± 0.00 ^{b}	0.09 ± 0.01 ^{a}	0.07 ± 0.01 ^{a}	0.06 ± 0.00 ^{b}	1.6 (47)
3-Methylbutyl acetate	0.50 ± 0.05 ^{a}	0.80 ± 0.14 ^{a}	0.65 ± 0.07 ^{a}	0.60 ± 0.03 ^{a}	1.0 (6)
Ethyl 2-phenylacetate	0.12 ± 0.00 ^{b}	0.26 ± 0.02 ^{a}	0.16 ± 0.12 ^{ab}	0.17 ± 0.01 ^{ab}	3.8 (6)
Ethyl acetate	11.30 ± 0.14 ^{a}	14.20 ± 2.97 ^{a}	11.75 ± 0.64 ^{a}	14.05 ± 0.07 ^{a}	30 (47)
Ethyl hexanoate	0.14 ± 0.01 ^{a}	0.13 ± 0.01 ^{a}	0.15 ± 0.01 ^{a}	0.14 ± 0.00 ^{a}	0.21 (47)
Ethyl octanoate	0.20 ± 0.01 ^{ab}	0.11 ± 0.01 ^{c}	0.15 ± 0.01 ^{bc}	0.23 ± 0.02 ^{a}	0.9 (6)
Ethyl decanoate	0.10 ± 0.00 ^{a}	0.03 ± 0.01 ^{c}	0.06 ± 0.01 ^{b}	0.09 ± 0.01 ^{a}	1.5 (47)
Fusel alcohols					
Propan-1-ol	19.5 ± 0.2 ^{b}	20.1 ± 4.1 ^{ab}	19.3 ± 1.3 ^{b}	27.0 ± 0.6 ^{a}	600 (6)
2-Phenylethanol	31.4 ± 1.7 ^{a}	32.5 ± 0.4 ^{a}	37.5 ± 1.7 ^{a}	35.0 ± 2.2 ^{a}	40-100 (6)
2-Methylpropan-1-ol	28.9 ± 0.5 ^{b}	43.9 ± 8.9 ^{a}	32.9 ± 2.1 ^{ab}	35.6 ± 0.0 ^{ab}	100 (6)
2,3-Methylbutan-1-ol	56.5 ± 0.2 ^{a}	76.7 ± 15.8 ^{a}	59.7 ± 3.8 ^{a}	60.2 ± 0.7 ^{a}	50 (6)
Fatty acids					
3-Methylbutanoic acid (isovaleric acid)	0.83 ± 0.06 ^{b}	0.72 ± 0.02 ^{b}	0.50 ± 0.03 ^{c}	1.15 ± 0.07 ^{a}	1.5 (18)
Hexanoic acid	1.55 ± 0.07 ^{ab}	1.10 ± 0.00 ^{c}	1.40 ± 0.00 ^{b}	1.75 ± 0.07 ^{a}	8 (6)
Octanoic acid	3.60 ± 0.14 ^{a}	2.40 ± 0.28 ^{b}	3.35 ± 0.21 ^{a}	3.75 ± 0.07 ^{a}	15 (6)
Decanoic acid	1.14 ± 0.23 ^{ab}	0.90 ± 0.28 ^{b}	1.02 ± 0.11 ^{b}	1.50 ± 0.00 ^{a}	10 (18)
Ketones					
Butane-2,3-dione (diacetyl), total	0.10 ± 0.01 ^{b}	0.07 ± 0.01 ^{bc}	0.05 ± 0.00 ^{c}	0.28 ± 0.00 ^{a}	0.1-0.14* (22)
Pentane-2,3-dione, total	< 0.01 ^{a}	< 0.01 ^{a}	< 0.01 ^{a}	< 0.01 ^{a}	0.9 (6)

Continued

Compound	*YC*	*MS*	*PreBWS*	*PostBWS*	Sensory threshold*
3-Hydroxybutan-2-one (acetoin)	2.2 ± 0.2 [b]	2.5 ± 0.1 [b]	1.4 ± 0.3 [c]	22.6 ± 0.2 [a]	50 (47)
Polysulfide					
Dimethyl sulfide free	< 0.01 [a]	< 0.01 [a]	< 0.01 [a]	< 0.01 [a]	0.03-0.045 (47)
Linear aldehyde					
Acetaldehyde	5.35 ± 0.49 [c]	40.25 ± 9.97 [a]	14.25 ± 0.49 [b]	14.05 ± 0.35 [b]	10 (18)

Each value was expressed as mean ± standard deviation analysed from two independent brews. For each compound, a different letter in each row denotes a significant difference at $P < 0.05$. Sensory threshold values retrieved from the literature referenced as superscript. * Sensory threshold for diacetyl.

The soured beers were perceived as clearly sour with untypical ("not pure") smells identified for *MS* and *postBWS*, while a slight plum aroma was described for *preBWS*. The control samples (*YC*) were described as having a pure and characteristic smell with slightly cider and plum aroma, while the taste was slim and typical in acidity. Apart from *postBWS* samples, which had an astringent aftertaste, the sour beers lingered with a fading sour aftertaste. Cell autolysis could have provoked the astringency, as well as influence the overall flavour profile by releasing both substrates (lipids, proteins, and carbohydrates) and intracellular enzymes (Bokulich and Bamforth, 2013). An elevated astringency derived from the prolonged contact time with husk during *MS* was not detected. In this regard, a low pH during mashing was found in the past to limit their extraction (Briggs et al., 2004).

6.5 Conclusion

Souring intended for sour beer production can be done at various time points along the brewing process, but the practice employed will ultimately influence attributes of both a technological and quality nature. The exposure of yeast cells to acidic conditions caused a delay in their growth and performance. Although this did not influence complete attenuation of sour beers, it might have impaired the re-absorption of off-flavours after primary fermentation, which primarily impacted the final quality of co-fermented samples. High levels of acetaldehyde and decreasing lautering rates were detected when acidification is done in the mash. Further processing of soured mash also led to a dilution of their initial acidity level. Overall, the practice of souring wort before boiling emerged as the best way to obtain a sour beer with high acidity and minimal organoleptic failures.

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Chapter 7: Growth study, metabolite development and organoleptic profile of a malt-based substrate fermented by lactic acid bacteria

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7.1 Abstract

The objective of this study is to investigate the fermentation of barley malt extract by four different lactic acid bacteria (LAB) species and to analyse the effect on biological, physicochemical and organoleptic attributes of the resulting beverages. Malt and wort were analysed following the Methodensammlung der Mitteleuropäischen Analysenkommission (MEBAK). Sugars and organic acids were evaluated using HPLC, while aroma compounds and ageing indicators were quantified using a headspace GC-FID (flame ionisation detector). Unhopped wort was able to support the growth of all LAB during the whole experiment time (120 h) with a cell count increase up to 3.6 log orders. The strain-specific preferences for carbon and nitrogen sources led to significant differences in the accumulation of metabolic by-products, i.e. organic acids and flavour-active compounds released in the medium. LAB fermentation lowered the amount of some Strecker-aldehydes (2-, 3-methylbutanal, 2-phenylacetaldehyde) carrying "malty" off-flavours and increased dairy-related aroma compounds such as diacetyl and acetoin ("buttery"). The fermented samples were sensorially distinct and described with "bready", "yeasty", "yogurty" or "honey" attributes. In general, LAB fermentation conferred flavour complexity to wort. Forced ageing of the fermented samples led to a large increase of "staling" indicators, but these samples improved organoleptic stability compared to the unfermented control.

7.2 Introduction

During the last few decades, the accumulation of scientific evidence linking nutrition and well-being has progressively steered the choices of the modern consumer towards healthier and natural food alternatives (Granato and Branco, 2010; Prado et al., 2008). This trend compels food companies to continuously work on novel formulations or adapt older ones to new standards (Eckert and Riker, 2007). The increase in "functionality" is a widespread and commercially attractive way to improve the potential health benefits of food products (Pihlant and Korhonen, 2015). Functional beverages in particular have recently been widely popularised in the form of flavoured waters, fruit juices, energy drinks and teas (Sorenson and Bogue, 2009).

Cereals carry of beneficial functions in form of dietary fibre (Anderson et al., 2001) and phytochemicals (e.g. sterols, phenolic acids) (Arendt and Zannini, 2013). They constitute therefore an attractive substrate for the development of novel, non-alcoholic and functional beverages. The production of cereal-based drinks is an interesting way to diversify the product portfolio for many breweries and to relieve them from any major economic losses due to the general decrease in beer consumption (The Brewers of Europe, 2014). The implementation of this technology does not normally require a radical change of the existing plant and can take advantage of any overcapacities present (Krahl, 2010).

Since unprocessed cereal-based products are often considered bland or unpleasant in flavour (Chavan et al., 1989), fermentation is often applied in order to improve the palatability (Leroy and De Vuyst, 2004; Nout, 2009). Lactic acid bacteria (LAB) can be used to increase the flavour complexity of these substrates. This heterogeneous group of Gram-positive lactic acid bacteria have a long history of use in food products and several of them have been granted GRAS status by the Food and Drug Administration (FDA). The use of LAB to ferment wort is applied to regulate the pH levels during the production of beer aimed to be manufactured according to the German purity law (Kunze, 2010). LAB fermentation gives rise to a wide range of volatile and non-volatile compounds, e.g. organic acids, alcohols, aldehydes, ketones and carbonyl compounds (McFeeters, 2004). Depending on the metabolic features of the starter culture, as well as the cereal substrate and the manufacturing processes involved, different strains of LAB may generate characteristic and contrasting end products (Blandino et al., 2003). The knowledge of which organoleptic-active compounds are being released during wort fermentation can help in the tailored formulation of novel, specialty "sour beers",

inspired from European styles such as the Belgian *Lambics* or the German *Berliner Weisse*, or from traditional fermented beverages (Waters et al., 2013). Malting of cereals helps to provide better conditions for sustaining LAB fermentation due to the higher amount of assimilable nutrients (Charalampopoulos et al., 2003) and contributes to the aroma profile of the substrate (Kunze, 2004).

Sensory evaluation plays an essential role in profiling the flavour attributes and assessing the final consumer acceptance of a novel product (Bredie and Moller, 2012). Maintaining the smell and flavour quality from bottling throughout the shelf-life of the product during storage represents an enormous challenge. Natural deterioration takes place due to chemical reactions, mechanical stress during transportation, light influence, thermal stress caused by inadequate storage conditions and by oxidative processes (Narziss, 2005). Over the course of the product shelf-life, cereal-based beverages commonly experience an accumulation of staling compounds and a decrease in positive organoleptic attributes (Vanderhaegen et al., 2006). For these reasons, the understanding of the evolution of the flavour profile during ageing is essential for the commercial success of a product. Numerous studies and reviews have been carried out on the origins of flavour deterioration in beer (Franz and Back, 2003; Saison et al., 2009; Suzuki et al., 2006; Vanderhaegen et al., 2007), but, to date, only one has considered the same problem for LAB-fermented cereal-based beverages (Krahl, 2010).

The main aim of this work is to study the suitability of barley malt-based wort to build a base for novel non-alcoholic beverages fermented by LAB. Biological, physicochemical and organoleptic changes were periodically studied for species of *Weissella cibaria*, *Lactobacillus brevis*, *Lb. reuteri* and *Lb. plantarum* and related to the strain-specific metabolism. The flavour profile of the fermented samples was compared before and after simulated ageing with the aim of understanding the main chemical transformations responsible for "staling".

7.3 Materials and methods

7.3.1 Bacterial cultures and reagents

The microorganisms used as inoculum in this study (Table 21) belong to the culture collection of the Cereal and Beverages Research Group of the University College Cork, Ireland. The LAB isolates were maintained as frozen stocks in 80% (w/w) glycerol tubes at -80°C. The strains were routinely sub-cultured on deMan Rogosa Sharp (MRS) plates (Oxoid, Basingstoke, Hampshire, England) under anaerobic conditions for 24 h at 30°C for *W. cibaria* PS2, *Lb. brevis* R2Δ, *Lb. plantarum* FST1.7 and 37°C for *Lb. reuteri* R29.

Table 21. Bacterial strains used in this study.

Species	Strain code	Metabolism	Growth temperature	Source
Weissella cibaria	PS2	Obl. heterofermentative	30°C	Sourdough
Lactobacillus brevis	R2Δ	Obl. heterofermentative	30°C	Porcine
Lactobacillus reuteri	R29	Obl. heterofermentative	37°C	Human
Lactobacillus plantarum	FST1.7	Fac. heterofermentative	30°C	Cereal

All reagents used in the following trials were at least analytical-grade from Sigma-Aldrich, Missouri, USA, unless otherwise stated. Malt and wort were analysed according to standard methods described by the Mitteleuropäische Brautechnische Analysenkommission (MEBAK) (MEBAK 2011a, MEBAK, 2012).

7.3.2 Barley malt and mashing regime

Commercial malt made from spring barley (*Hordeum vulgare*, variety *Propino*), harvested in 2012, was purchased from the Malting Company of Ireland Ltd. (Cork, Ireland), and stored in barrels at room temperature prior to use. The malt characteristics including the analytical methods used are listed in Table 22.

Table 22. Characteristics of raw barley and malt. The results were obtained from at least triplicates analysis. Values are presented as mean ± SD of triplicates.

Analysis	Method	Unit	Malt
Extract	MEBAK 3.1.4.2.2	% (dry basis)	83.8 ± 0.3
Saccharification	MEBAK 3.1.4.2.4	min	25 ± 5
Moisture	MEBAK 3.1.4.1	%	4.8 ± 0.1
Colour	MEBAK 3.1.4.2.8.2	EBC	3.6 ± 0.1
Filterability	MEBAK 3.1.4.2.5	h	1-2
Thousand kernel weight	MEBAK 1.3.2	g	40.4 ± 0.3
Total nitrogen	MEBAK 1.5.2.1	% (w/w) (dry basis)	1.68 ± 0.04
Total protein	Total Nitrogen x 6.25	% (w/w) (dry basis)	10.5 ± 0.3
Total soluble nitrogen	MEBAK 3.1.4.5.2.1	% (w/w) (dry basis)	0.65 ± 0.00
Kolbach index	MEBAK 3.1.4.5.3	% (w/w) (dry basis)	39 ± 0
Free amino nitrogen	MEBAK 3.1.4.5.5.1	mg/100 g malt (dry basis)	141 ± 12
pH	MEBAK 3.1.4.2.7	-	5.98 ± 0.01
Viscosity	MEBAK 3.1.4.4.1	mPa·s	1.513 ± 0.036
Friability	MEBAK 3.1.3.6.1	%	95 ± 0
Partly unmodified grain	MEBAK 3.1.3.6.1	%	2.5 ± 0.4
Glassy corns	MEBAK 3.1.3.6.1	%	1.9 ± 0.3
Limit of attenuation	MEBAK 2.8.2	%	70.6 ± 0.1

The malt was milled with a two-roller mill fitted with a 0.7 mm distance gap between the rollers. A pilot-scale (60 L) brewhouse comprising of a combined mash-boiling vessel, a lauter tun and a whirlpool tank was used for mash and wort production. A grist-to-liquor ratio of 1:3.5 was chosen. The following mashing regime was employed: 20 min at 50°C, 40 min at 62°C, 20 min at 72°C, and 5 min at 78°C for mashing-off. The heating rate was of 1°C/min between the temperature rests.

7.3.3 Wort production

The mash was pumped in the lauter tun and lautering was performed employing two sparging steps of 7 L each. Wort was boiled for 30 min for sterilisation purposes and adjusted to a final extract content of 6% (w/w) by adding boiling brewing water. Hot trub precipitates were removed by means of the whirlpool. The unhopped wort was then filled hot into 19 litre stainless steel containers and kept for a few days at 0°C.

7.3.4 Wort fermentation (beverage base production)

Tempered wort (4 L) was filled into 5 L sterile glass carboys in duplicate for each fermentation trial and for the unfermented control. Single colonies of each of the four LAB strains were propagated twice into 10 mL MRS broth in anaerobic and static conditions for 24 h at optimal temperature. After cell count determination by means of a spectrophotometer (Helios Gamma UV-Vis Spectrophotometer, Thermo Electron Corporation, England) at 600 nm, the suspensions were centrifuged and washed twice with Ringer's solution. The inoculation was performed at ca. 7 log cfu/mL directly into the wort and fermentation was performed anaerobically under dark and static conditions at optimum temperature. Samples were taken at 0 h, 24 h, 48 h, 72 h, and 120 h and assessed for viable cell count. The rest was stored at -20°C into 50 mL screw-cap tubes for analysis of total titratable acidity (TTA), pH, free amino nitrogen (FAN), organic acids and sugars. All analyses were performed as replicates from two independent fermentation trials.

7.3.5 Viable cell enumeration

Total cell counts (cfu/mL) of LAB were performed on MRS agar plates after incubation for 48 h under anaerobic conditions using Anaerocult A gas packs (Merck, Darmstadt, Germany) at optimal growing temperature.

7.3.6 Measurement of titratable acidity, pH and free amino nitrogen

Total titratable acidity (TTA) was assessed by titrating the samples with 0.25 M NaOH until pH 8.1 according to method 3.2.3 (MEBAK, 2011a). The pH was monitored using a commercial digital pH meter following method 2.13 (MEBAK, 2011a). The free amino nitrogen (FAN) concentration was determined as described in method 2.6.4.1.1 (MEBAK, 2011a) according to the Ninhydrin photometric method and using glycine as a control.

7.3.7 Determination of organic acids, sugars and ethanol

Organic acids and sugar concentrations were determined by high performance liquid chromatography HPLC (Agilent 1260 Infinity) equipped with both a diode array detector (DAD) and a refractive index detector (RID). All measurements were performed using an Agilent Hi-Plex H (7.7 x 300 mm, pack size 8 μm) column with a

PL Hi-Plex Guard column mounted upstream. For organic acids analysis, the samples were previously sterile filtered through 0.45 μm MINISART-plus filters (Sartorius Stedim Biotech GmbH, Goettingen Germany) and 20 μL of the filtrates were injected and eluted in an isocratic elution mode with a flow rate at 0.5 mL/min using 0.004 M H_2SO_4 as mobile phase. The column was kept at 65°C and the samples were detected during a period of 30 min by a diode array detector set at 210 nm. Samples intended for sugar analysis were previously clarified using Carrez I (3.6% w/v $K_4[Fe(CN)_6]$) and Carrez II (7.2% w/v $Zn(CH_2COO)_2 \bullet 2H_2O$) as described by Indyk et al. (1996), then sterile filtered. A volume of 20 μL was injected and eluted isocratically using a flow rate of 0.6 mL/min and MilliQ water as mobile phase. The column was kept at 25°C and the samples were detected by a refractive index detector. Each run lasted 20 minutes. External standards (lactic and acetic acids, maltotriose, maltose, glucose and fructose) with known concentrations were used to build standard curves in order to quantify the samples peak areas. The concentration of L-lactic acid was assessed using a commercial enzymatic kit (K-LATE, Megazyme International, Kildare, Ireland). The quantification was based on the stereospecific oxidation of L(+)-lactate catalysed by L-lactate dehydrogenase and the resulting reduction of nicotinamide-adenine dinucleotide (NAD+) to NADH, which increased the absorbance at 340 nm. Ethanol content of the samples was measured using an Alcolyzer Beer ME Analyzing System (Anton Paar GmbH, Graz, Austria).

7.3.8 Characterisation of the flavour profile, ageing compounds and sensory profile

Wort samples fermented for 48 h were collected and filtered through a pilot-scale depth filter device (depth filter sheets K200, Pall Corporation, USA) and aseptically filled in 330 mL brown bottles, followed by pasteurisation for 15 min at 65°C (Priorclave Tactrol 2, Priorclave Ltd., UK). Pasteurisation was applied to ensure microbiological stability and to prevent further acidification processes, as well as to reproduce a common manufacturing practice in commercial beverage production. Half of the samples were then subjected to forced ageing as reported by Eichhorn (1991). Briefly, the bottles were shook in an upright position for 24 h at a temperature of 20°C to simulate transportation conditions; afterwards they were kept in a dark 40°C warm storing shelf for 4 more days, corresponding to a natural ageing process of about three to four months. The fresh (i.e. not aged) samples were stored at 4°C instead. The fresh

and forced aged samples were scored against each other by a trained panel of ten persons based on a modified version of the DLG (Deutsche Landwirtschafts-Gesellschaft e.V.) tasting scheme. Five categories (smell, purity of taste, body, carbonation and quality of bitterness) were assessed using a 5-points scale (5 = very good to 1 = unsatisfactory), and smell and taste attributes were recorded as well. The final rating was calculated using the following equation (MEBAK, 2014):

$$\text{Rating} = \frac{2 \times \text{Smell} + 2 \times \text{Taste} + 1 \times \text{Body} + 1 \times \text{Carbonation} + 2 \times \text{Bitterness}}{8}$$

The taste stability score was calculated as the difference between the rating of fresh and forced aged samples.

The ageing indicators were assessed prior to and after forced ageing according to the MEBAK method 2.23.4, whereas analysis of the fatty acids and fermentation by-products (e.g. aroma compounds) were done solely on the fresh samples. Briefly, the volatile ageing substances (MEBAK, 2011a) were firstly separated from the sample through steam distillation and subsequently extracted in dichloromethane after phase separation by centrifugation. Concentration of the organic phase was performed in a stream of nitrogen gas, and co-eluting acids were removed with ammonia. Finally, the cleaned organic phase was injected in a capillary gas chromatograph equipped with two flame ionisation detectors (FID). Steam-volatile aroma compounds (method 2.23.6) were analysed as above, but potassium hydrogen sulphite was added prior to extraction to remove carbonyl compounds which might interfere with the analysis (method 2.23.6). Highly volatile fermentation by-products, assessed following method 2.21.1, were consisting mainly of higher alcohols and esters and the analysis involved a headspace method coupled with GC-FID. Vicinal diketones (method 2.21.5.1) and acetoin (method 2.21.5.4) were measured using a headspace gas chromatograph coupled with an electron capture detector (ECD). Bound sulphur dioxide was liberated with phosphoric acid and heat application, then collected in a receiver containing hydrogen peroxide and the formed sulphuric acid was titrated with sodium hydroxide according to method 2.21.8.2. Free dimethyl sulphide was determined using a headspace gas chromatograph equipped with a sulphur-specific detector as described in method 2.23.1.1. All analyses were done in duplicates.

7.3.9 Statistical analyses

One-way ANOVA on Minitab software (Version 17.0) was used to compare the means of single aroma compounds and ageing indicators between the different treatments. When F-values were found significant, Tukey's multiple comparisons procedure was used to further determine any significant differences between the ferments. A two-sample paired t-test was used to compare averages between fresh and forced aged samples and was represented as a percentage change (symbol Δ). The level of significance was determined at $P < 0.05$. Results are expressed as mean ± standard deviation.

7.4 Results and discussion

7.4.1 Cell growth, pH, total titratable acidity and free amino nitrogen

The aim of this study was to monitor and compare the growth profile and the metabolite production of strains of *W. cibaria*, *Lb. brevis*, *Lb. reuteri* and *Lb. plantarum* when inoculated in barley malt wort. Each wort sample was inoculated with approximately the same LAB cell concentration (6.8-6.9 log cfu/mL) at time 0 h and samples were taken at defined time intervals to study relevant parameters. Results from the growth curves (Figure 17A) showed that all strains reached a maximal cell concentration within the first 24 h and 48 h of fermentation. Differences in the strain metabolism and adaptability to the substrate were responsible for contrasting outcomes. The strain *W. cibaria* PS2 showed the weakest growth among the tested strains reaching a maximal cell concentration of ca. 8.1 log cfu/mL after 24 h before rapidly entering the decline phase and maintaining a stable cell count (6.8 log cfu/mL) for the rest of the fermentation. The most vigorous growth was recorded with *Lb. plantarum* FST1.7, which extended the stationary phase up to 72 h, reaching a maximal cell concentration of 10.5 log cfu/mL after 48 h of fermentation. Both *Lb. brevis* R2Δ and *Lb. reuteri* R29 showed a similar growth behaviour, achieving ca. 9.5 log cfu/mL within the first 48 h, whereas no growth was observed in the uninoculated wort for the entire period of the trial. The largest decrease in pH concurred with the exponential growth phase of the strains and generally recorded a 2-points reduction within the first 24 hours, starting from an initial value of pH 5.69-6.14 and ending at pH 3.54-4.14 (Figure 17B). The best grower in this study, *Lb. plantarum* FST1.7, reached also the lowest pH value (pH 3.24 after 120 h) and at the same time the highest titratable acidity (40.8 ± 0.4 mmol/L) (Figure 17C). In comparison, *W. cibaria* PS2 and *Lb. brevis* R2Δ produced only about half the amount of titratable acids, whereas *Lb. reuteri* R29 accumulated 35.3 ± 0.4 mmol/L.

The cell cultures analysed in this study originated from diverse environmental sources, and differences in adaptability in the cereal substrate were therefore expected. However, strains sourced from cereal environments were not necessarily performing better, as in the case of *W. cibaria* PS2 which was isolated from sourdough. The weak growth of some strains could also be explained by the different intrinsic tolerance against high acidity levels (Passos et al., 1993). Species of *Lb. plantarum* have been already found to grow well at very acidic conditions (Charalampopoulos et al., 2003; G-Alegria et al., 2004) due to their ability to better control large pH gradients between the cytoplasma

and the external environment (Kashket, 1987; McDonald, 1990). The ability to withstand better low pH levels by *Lb. plantarum* FST1.7 was confirmed in growth trials in pH-corrected barley malt wort (results not shown). In this experiment, *W. cibaria* PS2 stopped the growth at pH 4.0, while *Lb. brevis* R2Δ and *Lb. reuteri* R29 managed to grown until pH 3.5. Finally, *Lb. plantarum* FST1.7 was the only strain able to grow until a pH of 3.0. Similarly, Passos et al. (1993) found that during acidification of cucumber juice*, Lb. plantarum* continued to grow until the pH decreased as low as 3.37.

LAB strains are nutritionally demanding microorganisms and typically require essential micronutrients, i.e. metal ions, vitamins, nucleotide and essential amino acids, for substantial growth as they are limited in synthesizing their own growth factors (Axelsson, 1998). Nevertheless, in past studies, barley malt extract has shown to well sustain the growth of LAB (Krahl, 2010; Patel et al., 2004; Salmerón et al., 2009). Charampopoulous (2002) showed that barley malt sustained better growth of LAB strains than raw barley and wheat because of the higher amount of sugars and free amino nitrogen available. In the same study, the author found that *Lb. plantarum* spp. and *Lb. reuteri* spp. have reached cell concentrations in the order of 10.1 and 8.9 log cfu/mL, respectively, after 12 h when inoculated into the wort medium. A generous source of organic nitrogen that can be easily assimilated by the bacterial cells is also crucial for a good fermentation performance, as LAB are limited in synthesizing *de novo* amino acids (Endo and Dicks, 2014). Generally, the amino acids required by all LAB, L-glutamic acid, L-isoleucine, L-leucine and L-valine, are also present in sufficient concentrations in all-malt wort (10°P) (Boulton and Quain, 2001a). The free amino nitrogen (FAN) source measured in this study gives an estimate of the amount of amino acids, ammonia and peptides with a terminal α-amino nitrogen group. From an initial value of 89.6 mg/L FAN, the overall consumption ranged from 19.4 to 28.8 mg/L after 120 h (Figure 17D). The uptake of this nitrogen fraction reflected the capacity of the single strains to dominate wort and confirmed its importance as an essential factor for growing cell cultures. A decrease in FAN concentration was observed, however, also when bacterial cultures entered the stationary and decline phase. Additionally, the unfermented control confirmed a slight but continuous decline in FAN concentration during the whole fermentation trial (results not shown). This suggests that the nitrogen fractions were participating in other reactions not involved with bacterial growth, e.g. non-enzymatic Maillard reactions. The apparent increase in the FAN values found for *Lb. reuteri* R29 between 24 h and 48 h were not significantly different ($P < 0.05$). A

positive proteolytic activity of this strain was tested *in vitro* using milk agar plates (Appendix 1). The high final FAN concentration indicated that the nitrogen source was not a limiting factor for any of the studied fermentation, as it was seen also in other similar works (Charalampopoulos et al., 2002; Charalampopoulos et al., 2003).

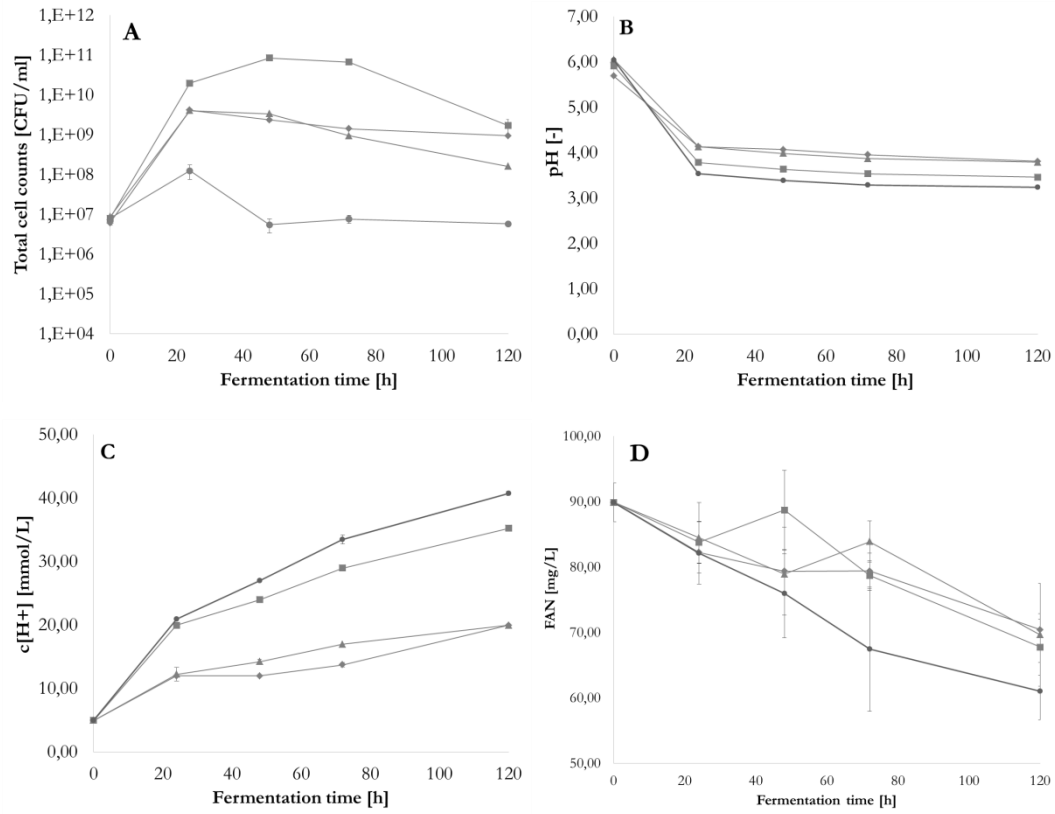

Figure 17. Evolution of cell population (A), pH (B), titratable acids (C) and free amino nitrogen (D) during the fermentation of barley malt extract (6% (w/w) extract content). *W. cibaria* PS2 (♦), *Lb. brevis* R2Δ (▲), *Lb. reuteri* R29 (■) and *Lb. plantarum* FST1.7 (●). Values are presented as mean of duplicates from two independent experiments. Error bars indicate standard deviation.

7.4.2 Sugar consumption profile

The four fermentable sugars considered during the trials and their initial concentrations were maltose (29.51 ± 0.64 g/L), maltotriose (7.88 ± 0.23 g/L), glucose (4.29 ± 0.08 g/L) and fructose (0.66 ± 0.12 g/L). The concentrations match the values found for barley malt extract (Narziss, 2009). Maltose was in this study the most abundant carbon source present, but it did not contribute always as the main energy source. Instead, each strain showed a specific preference for one or more sugar. Depending on how hexoses are fermented, LAB can be broadly divided into three groups. Obligately homofermentative LAB ferment glucose mainly via the glycolytic pathway (or Embden-

Meyerhof-Parnas pathway), yielding almost exclusively lactic acid (> 90%) (Endo and Dicks, 2014). The phosphoketolase pathway found in obligately heterofermentative LAB can theoretically yield lactic acid, ethanol and CO_2 in an equimolar ratio from the consumption of one mole of glucose. However, if additional substrates such as fructose or oxygen are present to restore the "redox-balance", they can serve as alternative electron acceptor, and acetate can be produced instead of ethanol (Kandler, 1983). This reaction is particularly important for heterofermentative LAB, as double the amount of energy in the form of ATP can be gained by the cell if acetate is built rather than ethanol. Finally, facultatively heterofermentative LAB strains have the ability to switch between heterolactic and homolactic fermentation mode, depending upon the carbon source, although glucose is exclusively fermented to lactic acid via glycolysis (Endo and Dicks, 2014).

As it can be seen in Figure 18, the consumption peak was registered within the first 48 h. The concentrations profile suggested that both *W. cibaria* PS2 and *Lb. plantarum* FST1.7 used glucose as their main source of energy, with the latter strain depleting also fructose after less than 48 h of fermentation. The preference for monosaccharide utilisation by the facultatively heterofermentative species of *Lb. plantarum* was also observed in other studies (Charalampopoulos et al., 2002; Gobbetti et al., 1994). The strain metabolised hexoses using the glycolytic pathway (homolactic fermentation) as being the most rewarding in terms of energy gain, which could partially justify the better growth performance and adaptability of this strain during the study. The disaccharide maltose was seen to be the preferred source of energy for *Lb. reuteri* R29. Many LAB species carry an intracellular maltose phosphorylase enzyme able to split maltose units into two glucose subunits, one phosphorylated and one not (Stolz et al., 1996). The latter is normally released into the medium again in order to avoid excessive intracellular concentrations. This could explain the simultaneous decrease in maltose and increase in glucose for both *Lb. brevis* R2Δ and *Lb. reuteri* R29. This matched the observations reported by Helland et al. (2004), which found that a *Lb. reuteri* strain used maltose as the primary carbohydrate supply and at the same time failed to use fructose when fermenting a maize flour and barley malt porridge. Studies by Stolz et al. (1995) confirmed the inability of *Lb. reuteri* to use fructose in sourdough medium. The increase in fructose concentration suggested the breakdown of sucrose molecules into their monomers. No significant consumption pattern was found for *Lb. brevis* R2Δ, however, preliminary tests done on single carbohydrate substrates in synthetic nutrient broth

confirmed that this strain was principally feeding on maltose, glucose and fructose substrate. Finally, maltotriose was not used significantly from any of the LAB tested. The consumption profile of sugars by the LAB cultures can have deep implications in the final sweetness intensity perceived by the consumer, as glucose and maltose have a perceived sweetness of one-half and one-third, respectively, compared to fructose (Moskowitz, 1970). Despite the heterofermentative metabolism of the strains involved, the highest ethanol level reached (0.16% (v/v) with *Lb. reuteri* R29) after 48 h fermentation was lower than the 0.5% (v/v) limit for non-alcoholic beverages (Kreisz et al., 2008).

Sugars were consumed along the whole fermentation period, even when the cell cultures stopped growing. Passos et al. (1994) postulated that the energy is required to further support the energy expenditure for maintaining cell viability under unfavourable conditions. Additionally, the final high residual concentrations suggested that sugars are not necessarily the growth-limiting factor. It is plausible that the pH and other metabolic by-products weakened and finally ceased the microbial growth (Charalampopoulos et al., 2002; Tenge, 2002).

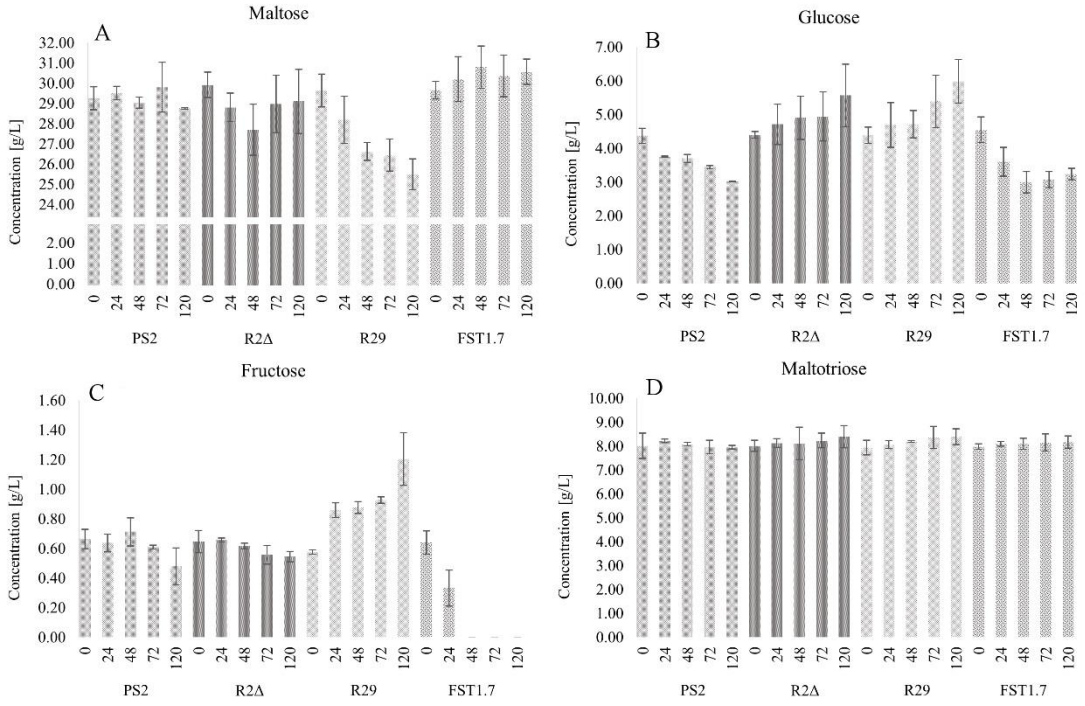

Figure 18. Consumption of maltose (A), glucose (B), fructose (C), and maltotriose (D) during fermentation of barley malt extract (6% (w/w) extract content) over a period of 120 h. PS2 (*W. cibaria*), R2Δ (*Lb. brevis*), R29 (*Lb. reuteri*) and FST1.7 (*Lb. plantarum*). Values are presented as mean of duplicates from two independent experiments. Error bars indicate standard deviation.

7.4.3 Organic acids

The concentrations of lactic and acetic acids (Figure 19) increased continuously over the fermentation period for all the variants, even during the stationary and decline phases of the strains, although the rate of accumulation was drastically reduced after the exponential phase. The combined values correlated with the titratable acidity measured for each sample. *Lb. plantarum* FST1.7 was the highest producer of lactate, with a concentration of 2.49 ± 0.21 g/L after 48 h, confirming the partial homolactic nature of the strain. Of this amount, 43% (w/w) were found to be L(+)-lactic acid, which is nutritionally more relevant than the D(-)-stereoisomer. The lowest amount of lactic acid was registered with *W. cibaria* PS2 (0.91 ± 0.19 g/L), followed by *Lb. brevis* R2Δ and *Lb. reuteri* R29, which released 1.14 ± 0.01 g/L and 1.61 ± 0.02 g/L lactic acid, respectively. All strains produced acetic acid as a result of heterolactic metabolism. The three obligate heterofermentative strains showed a relationship of acetic acid and lactic acid (AA:LA) in the range of 1:2.8 to 1:4.7, whereas the facultative heterofermentative *Lb. plantarum* FST1.7 released almost 12 times more lactic acid over acetic acid. The different relationship between these two organic acids may play an important role on the final organoleptic properties. Acetic acid has been reported having a "vinegar" sour note, conveying a more pungent sourness to the fermentate, while lactic acid has been regarded milder in the perceived acidity (Parker, 2012). Through a controlled oxygen input during fermentation, it would be possible to modulate the ratio of bacterial acetic and lactic acid released in the media towards preferred specifications (Bobillo and Marshall, 1991; Condon, 1987; Kandler, 1983). In this regard, oxygen could have been replenished in the headspace of the bottles during opening and sampling and therefore partially explaining the continuing increase in acetic acid for *Lb. plantarum* FST1.7 after depletion of fructose. The same is valid for any step controlling the release of fructose in the fermentable substrate. A study conducted by Rocken et al. (1992) showed that the presence of fructose in the form of invert sugar had a positive linear effect on acetate content of sourdough during baking applications.

7.4.4 Sensory profile

The DLG-tasting scheme was primarily applied for the organoleptic evaluation of beer, but the same approach can also be applied to other fermented malt-based beverages to identify typical flavour characteristics and evident off-flavours.

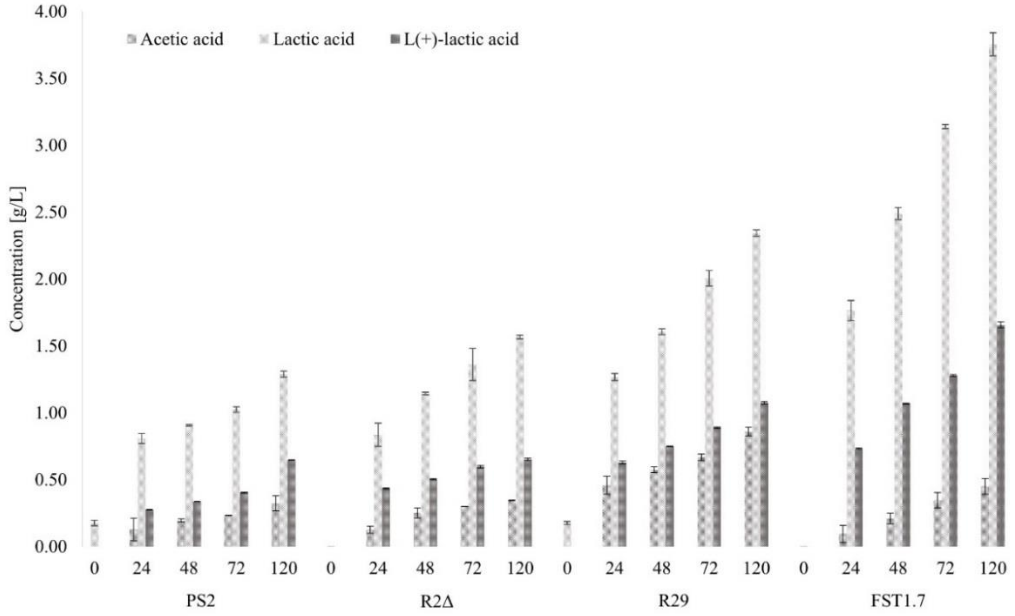

Figure 19. Concentration of acetic acid, lactic acid and L(+)-lactic acid during fermentation of barley malt extract (6% (w/w) extract content) over a period of 120 h. PS2 (*W. cibaria*), R2Δ (*Lb. brevis*), R29 (*Lb. reuteri*) and FST1.7 (*Lb. plantarum*). Values are presented as mean of duplicates from two independent experiments. Error bars indicate standard deviation.

In a study conducted by Krahl et al. (2009), the authors demonstrated the suitability of the DLG tasting scheme to describe malt-based beverages fermented by *Lactobacillus* spp.. The sensory of fresh (i.e. not aged, FS) and forced aged (FAS) samples are reported in Table 23. From the evaluation of each criterion, a weighted average score was formed.

Among the "fresh" samples, the unfermented wort control scored the best (4.09), followed by *Lb. reuteri* R29 (3.69) and *Lb. plantarum* FST1.7 (3.69), and finally *W. cibaria* PS2 (3.49) and *Lb. brevis* R2Δ (3.49). Forced ageing (FAS) caused a deterioration of both smell and taste attributes, with the unfermented wort control being this time the least stable product and considered "dull" and "lifeless". Both *Lb. reuteri* R29 and *Lb. plantarum* FST1.7 fermented worts showed a remarkable resistance to the ageing treatment, and were assigned with the highest final score of 3.69 respectively 3.56. The fermented samples of *Lb. reuteri* R29 evolved from a merely sour drink when fresh to a honey-rich and pungent one when forced-aged. The pronounced acidity perception might be attributed to the higher amount of acetic acid released by this strain during fermentation. The flavour threshold value for acetic acid in beer has been reported to be 0.20 g/L (Eckert and Riker, 2007; Gobbetti et al., 1994), thus significantly lower than the 0.53 ± 0.02 g/L acetate present in the drink. The samples treated with *Lb. plantarum*

FST1.7 conferred "honey" and "lactic" notes when fresh and evolved into "yogurt" tones when aged. The strain *Lb. brevis* R2Δ delivered wort with "meaty" and "yeasty" off-flavours, whereas *W. cibaria* PS2 ferment was classified as "bready" when fresh and "kvass-like" when aged. Both beverages showed little resistance to ageing, which was confirmed by the lowest score for taste stability (3.19 respectively 3.16). Release of carbon dioxide due to heterofermentative metabolism of LAB was not enough to be detected during tasting. In general, quality of bitterness worsened after forced ageing, but no changes were detected in the mouthfeel sensation of the drinks.

The results suggested that a lower pH in the fermented samples might help to preserve the smell and taste attributes during forced ageing. This assumption contradicts past studies done on beer flavour deterioration, which theorised that low pH was responsible in many cases for detrimental effects on the aroma of aged beer samples (Gijs, 2002; Hashimoto, 1972; Kaneda et al., 1997; Lermusieau et al., 1999).

Table 23. Sensory scores of fresh (FS) and forced-aged (FAS) according to DLG tasting scheme fermented with four different lactic acid bacteria (*W. cibaria* PS2, *Lb. brevis* R2Δ, *Lb. reuteri* R29 and *Lb. plantarum*) at optimal temperature for 48 h.

Parameter	Control		PS2		R2Δ		R29		FST1.7	
	FS	FAS	FS	FAS	FS	FAS	FS	FAS	FS	FAS
Smell	4.7	3.8	3.8	3.3	3.8	3.3	4	4.1	4.2	4
Purity of taste	4.7	3.8	3.8	3.3	3.8	3.3	4.2	4.1	4.2	4
Smell and taste descriptor	pure, honey	aged, dull	rye-bread	sour, kwass, plums	soya sauce	yeast, cider	sour	honey, pungent	butter, honey	yogurt, sour
Body	4.5	4.5	4.3	4.3	4.3	4.3	4.5	4.5	4.3	4.3
Carbonation	0	0	0	0	0	0	0	0	0	0
Quality of bitterness	4.7	4	4.2	3.9	4.2	4	4.3	4.3	4.2	4.1
Rating	4.09	3.46	3.49	3.16	3.49	3.19	3.69	3.69	3.69	3.56
Taste stability	0.63		0.33		0.30		0.00		0.13	

7.4.5 Aroma profile

In addition to the flavour-active compounds created at the early stages during the production of cereal malt-beverages, e.g. Maillard compounds formed during the kilning and boiling steps (Boulton and Quain, 2001a), metabolites formed biologically during LAB fermentation can impart either a positive contribution or an undesirable one. Generally, a well-equilibrated amount between all the flavour compounds will finally determine the overall beneficial contribution to the final aroma profile. Table 24 reports the concentrations of aroma-active compounds originating from fermentative activities of the different strains. None of the esters analysed significantly increased their value after LAB fermentation, and many of them were found to be under the detection limit of the method. These compounds represent the most important flavour-active by-product of yeast metabolism during beer fermentation and are mainly responsible for the fruity and flowery sensations (Hughes, 2008). Nevertheless, past studies revealed the capacity of *Lactobacillus* spp. to release esters during malolactic fermentation of red wines (Maicas et al., 1999; Pozo-Bayón and G-Alegria, 2005). Pyruvate plays a central role as an intermediate compound in the LAB catabolism of sugars as well as a substrate for the following production of various aromatic compounds such as diacetyl, acetoin, acetaldehyde or acetic acid (Van Kranenburg et al., 2002; Pastink et al., 2008). In this study, *Lb. plantarum* FST1.7 was found to release significantly higher ($P < 0.05$) amounts of diacetyl (0.17 ± 0.00 mg/L), acetoin (6.90 ± 0.00 mg/L) and acetaldehyde ("fuity, apple-like") (3.85 ± 0.35 mg/L) in the medium. The first two compounds are carriers of dairy-related notes, such as "buttery" and "lactic", and were identified also during the sensory tests. All three compounds were also found in *togwa*, a traditional Tanzanian cereal-based beverage fermented with LAB, e.g. *Lb. plantarum* spp, and yeasts (Mugula et al., 2003). Some LAB species can release these volatiles as a result of the citrate catabolism (Benito de Cardenas et al., 1989; García-Quintáns et al., 2008; Hickey et al., 1983; Keenan and Lindsay, 1968), which has been naturally found in malt-base wort (12°P) with levels up to 170 mg/L (Mandl, 1974). The low organoleptic threshold of diacetyl (0.05-0.14 mg/L) (Boulton and Box, 2003) makes it a common and undesirable off-flavour in beer.

The data showed a significant accumulation ($P < 0.05$) of propan-1-ol (2.25 ± 0.07 µg/L) by *Lb. reuteri* R29. This compound can impart a warming and "alcoholic" aroma and flavour to the substrate (Hughes, 2008). It is commonly produced by yeast cells together with other fusel alcohols during amino acids catabolism via the Ehrlich

pathway (Hazelwood et al., 2008). Although the authors could not find direct evidence supporting the accumulation of propan-1-ol by this LAB strain, Valdez et al. (1997) found that *Lb. reuteri* CRL 1100 was able to accumulate 1,3-propanediol, a precursor molecule to propan-1-ol, when grown anaerobically in a maltose/glycerol media. Isovaleric and hexanoic acid ("cheesy") were the only fatty acids which showed a significant decrease and increase ($P < 0.05$), respectively, due to fermentative activities.

Finally, fermentation by *Lb. brevis* R2Δ accumulated 1.01 ± 0.13 mg/L 3-methylbutyl acetate (= isoamyl acetate) (flavour threshold of 1.0 mg/L) (Bamforth, 2006), a volatile ester commonly found in beer as a result of yeast metabolism, and known to impart a "fruity" and "banana" aroma (Boulton and Quain, 2001b). A corresponding detection during sensory trials, however, was not found.

Table 24. Fermentation by-products [mg/L] analysed in fresh control and LAB-fermented wort.

Compound	Control	PS2	R2Δ	R29	FST1.7
Esters					
Ethyl butanoate	< 0.01 [a]	< 0.01 [a]	< 0.01 [a]	< 0.01 [a]	< 0.01 [a]
2-Methylpropyl acetate	< 0.01 [a]	< 0.01 [a]	< 0.01 [a]	< 0.01 [a]	< 0.01 [a]
Ethyl-2-phenylacetate	< 0.01 [a]	< 0.01 [a]	< 0.01 [a]	< 0.01 [a]	< 0.01 [a]
Ethyl hexanoate	< 0.01 [a]	< 0.01 [a]	< 0.01 [a]	< 0.01 [a]	< 0.01 [a]
Ethyl octanoate	< 0.01 [a]	< 0.01 [a]	< 0.01 [a]	< 0.01 [a]	< 0.01 [a]
3-Methylbutyl acetate	0.10 ± 0.01 [a]	0.11 ± 0.01 [a]	1.01 ± 0.13 [a]	0.12 ± 0.00 [a]	0.12 ± 0.00 [a]
Ethyl acetate	< 0.1 [a]	< 0.1 [a]	< 0.1 [a]	< 0.1 [a]	< 0.1 [a]
Fusel alcohols					
Propan-1-ol	< 0.5 [b]	< 0.5 [b]	< 0.5 [b]	2.25 ± 0.07 [a]	< 0.5 [b]
2-Methylpropan-1-ol	< 0.5 [a]	< 0.5 [a]	< 0.5 [a]	< 0.5 [a]	< 0.5 [a]
2-, 3-Methylbutan-1-ol	1.25 ± 0.21 [a]	1.00 ± 0.00 [a]	1.25 ± 0.21 [a]	1.05 ± 0.07 [a]	1.05 ± 0.07 [a]
2-Phenylethanol	0.13 ± 0.00 [c]	0.19 ± 0.00 [b]	0.20 ± 0.00 [b]	0.23 ± 0.01 [a]	0.20 ± 0.01 [b]
Fatty acids					
3-Methylbutanoic acid (isovaleric acid)	0.52 ± 0.00 [a]	0.27 ± 0.01 [c]	0.25 ± 0.00 [c]	0.32 ± 0.00 [b]	0.27 ± 0.01 [c]
Hexanoic acid	0.12 ± 0.01 [c]	0.21 ± 0.00 [ab]	0.24 ± 0.01 [a]	0.22 ± 0.01 [ab]	0.21 ± 0.01 [b]
Octanoic acid	< 0.01 [a]	< 0.01 [a]	0.02 ± 0.00 [a]	< 0.01 [a]	< 0.01 [a]
Decanoic acid	< 0.01 [a]	< 0.01 [a]	< 0.01 [a]	< 0.01 [a]	< 0.01 [a]
Ketones					
Butane-2,3-dione (diacetyl), total	0.03 ± 0.00 [b]	0.01 ± 0.00 [b]	0.01 ± 0.00 [b]	0.01 ± 0.00 [b]	0.17 ± 0.00 [a]
Pentane-2,3-dione, total	< 0.01 [a]	< 0.01 [a]	< 0.01 [a]	< 0.01 [a]	0.01 ± 0.00 [a]
3-Hydroxybutan-2-one (acetoin)	0.90 ± 0.14 [b]	0.60 ± 0.00 [c]	0.60 ± 0.00 [c]	0.60 ± 0.00 [c]	6.90 ± 0.00 [a]
Polysulphide					
Dimethylsulfide free (μg/L)	< 10 [a]	< 10 [a]	< 10 [a]	< 10 [a]	< 10 [a]
Linear aldehydes					
Acetaldehyde	< 0.01 [c]	< 0.01 [c]	< 0.01 [c]	0.44 ± 0.04 [b]	3.85 ± 0.35 [a]

Each value was expressed as mean ± standard deviation analysed in duplicate from two independent fermentations.

[a-c] For each compound, a different superscript in each row denotes a significant difference at $P < 0.05$.

7.4.6 Ageing indicators

Ageing can be described as a dynamic process during which aroma and flavour compounds can be newly formed, increased or decreased (Eichhorn, 1991). Ageing indicators in cereal-based beverages are normally attributed to thermal treatments, oxygen exposure and/or as by-products of fermentation (Narziss, 2005). Relative changes in the concentrations prior and after forced-ageing allow the quantification of the flavour stability of the product (Table 25).

In most cases, forced ageing led to an accumulation of staling indicators regardless of the applied strain or treatment. Even though some of the compounds were not exceeding the sensory threshold (STH) in the fresh and aged variants, positive synergies between aroma-active compounds exist and have to be considered, especially during development of novel beverages. In a study conducted by Herrmann et al. (2010), 2- and 3-methylbutanal have been found to lower their respective sensory threshold (156 and 56 μg/L, respectively) to approximately one-fifth of their initial values when simultaneously present in beer. The flavours associated with these two compounds are described as "malty" and "roasty" (Saison et al., 2009). Both aldehydes may be the results of Strecker degradation of the branched-chain amino acids isoleucine and leucine, respectively (Vanderhaegen et al., 2003), or a strain-dependent conversion catabolised by pyridoxal-5'-phosphate-dependent aminotransferases (Van Kranenburg et al., 2002). In an similar way, the aromatic compound 2-phenyalcetaldehyde could have been formed from the conversion of the amino acid phenylalanine (Nierop Groot and de Bont, 1998). This molecule was found at significantly lower levels in the LAB-treated samples but its concentration increased during forced ageing, especially for *W. cibaria* PS2 (128.0 ± 4.2 μg/L). The "kwass"-like odour found in this sample could have been partially attributed to the "malty" note given by this compound. Fermentation by LAB was able to reduce the concentrations of the Strecker aldehydes compared to the unfermented control. This could be caused by the decreased substrate, i.e. sugars and amino acids, available for their formation, which may have been used as nutrients by the microbial cells. Krahl et al. (2010) found also that the amount of Strecker-aldehydes initially present in malt extract wort (7°P) decreased during the first 64 h of fermentation by *Lb. amylolyticus* spp., proposing it as a potential tool for increasing sensorial attributes in malt-fermented beverages. The same author observed that after LAB fermentation activity slowed down, the sum of the Strecker aldehydes increased again due to the thermal load. The low concentration of the analysed esters suggests that

this group plays a secondary role in LAB flavour development. Both thermal deterioration indicators 2-furfural ("caramel, bready") and γ-nonalactone ("coconut", "rancid") were found to accumulate after forced ageing in the fermented samples. Even though some fermented variants accumulated γ-nonalactone at concentrations much higher than its sensory threshold of 11.2 μg/L (Suzuki et al., 2006), no evident correlation was observed during sensory analysis. Guyot-Declerk et al. (2005) reported experiments on the perception of γ-nonalactone in beer depending on the pH level (pH 4.2 and 4.6). They found that the intensity of the coconut-note perceived in aged beers was greater when the pH was higher, which could partially explain an organoleptic absence in the more acidic LAB samples.

The comparison of the total ageing indicators (staling, oxygen and thermal indicators) showed that LAB fermentation was able to generally reduce the compounds responsible for ageing during fermentation. The subsequent accelerated ageing, however, led to a higher increase of these attributes in fermented samples compared to the unfermented control. Although it was seen that pH plays a major role in the development of Maillard products (Martins et al., 2000), many other factors could have played a role in the different distribution of ageing indicators (substrate depletion, reducing conditions, etc.).

Table 25. Levels of ageing indicators [µg/L] and their relative variation between fresh (FS) and forced aged (FAS) unfermented and fermented samples with four different lactic acid bacteria (*W. cibaria* PS2, *Lb. brevis* R2Δ, *Lb. reuteri* R29 and *Lb. plantarum* FST1.7) at optimal temperature for 48 h. The column Δ reports the concentration increment in % of the ageing indicators after forced altering.

Compound	Control			PS2			R2Δ			R29			FST1.7		
	FS	FAS	Δ	FS	FAS	Δ	FS	FAS	Δ	FS	FAS	Δ	FS	FAS	Δ
Aldehydes															
2-Methylbutanal	15.5 ± 0.7 ^{a}	21.0 ± 0.0 ^{a}	35	6.0 ± 0.0 ^{c}	15.0 ± 0.0 ^{b}	150*	6.0 ± 0.0 ^{c}	9.0 ± 0.0 ^{d}	50*	8.0 ± 0.0 ^{b}	10.5 ± 0.7 ^{c}	31	6.0 ± 0.0 ^{c}	8.0 ± 0.0 ^{d}	33
3-Methylbutanal	38.0 ± 1.4 ^{a}	56.0 ± 0.0 ^{a}	47*	10.5 ± 0.7 ^{d}	44.5 ± 0.7 ^{b}	324*	10.0 ± 0.0 ^{d}	22.0 ± 1.4 ^{d}	120*	26.5 ± 0.7 ^{b}	35.0 ± 0.0 c	32*	14.0 ± 0.0 ^{c}	22.0 ± 1.4 ^{d}	57
Benzaldehyde	< 5.0 ± 0.0 ^{a}	5.0 ± 0.0 ^{a}	-	< 5.0 ± 0.0 ^{a}	5.5 ± 0.7 ^{a}	10	< 5.0 ± 0.0 ^{a}	< 5.0 ± 0.0 ^{a}	-	< 5.0 ± 0.0 ^{a}	< 5.0 ± 0.0 ^{a}	-	< 5.0 ± 0.0 ^{a}	5.0 ± 0.0 ^{a}	-
2-Phenylacetaldehyde	113.5 ± 3.5 ^{a}	132.0 ± 9.9 ^{a}	16	25.5 ± 3.5 ^{bc}	128.0 ± 4.2 ^{a}	402*	20.0 ± 2.8 ^{c}	65.0 ± 1.4 ^{b}	225*	49.5 ± 7.8 ^{b}	84 ± 1.4 ^{b}	70	34.5 ± 10.6 ^{bc}	70.0 ± 2.8 ^{b}	103
Esters															
Succinic acid diethyl ester	5.0 ± 0.0 ^{a}	5.0 ± 0.0 ^{b}	0	5.0 ± 0.0 ^{a}	6.0 ± 0.0 ^{a}	20	6.0 ± 0.0 ^{a}	5.0 ± 0.0 ^{b}	-17	5.5 ± 0.7 ^{a}	5.0 ± 0.0 ^{b}	-9	5.5 ± 0.7 ^{a}	5.0 ± 0.0 ^{b}	-9
Nicotinic acid ethyl ester	< 5.0 ± 0.0 ^{a}	< 5.0 ± 0.0 ^{b}	-	< 5.0 ± 0.0 ^{a}	9.5 ± 0.7 ^{a}	90	< 5.0 ± 0.0 ^{a}	9.5 ± 0.7 ^{a}	90	< 5.0 ± 0.0 ^{a}	< 5.0 ± 0.0 ^{b}	-	< 5.0 ± 0.0 ^{a}	< 5.0 ± 0.0 ^{b}	-
Phenylacetic acid ethyl ester	< 5.0 ± 0.0 ^{a}	< 5.0 ± 0.0 ^{a}	-	< 5.0 ± 0.0 ^{a}	< 5.0 ± 0.0 ^{a}	-	< 5.0 ± 0.0 ^{a}	< 5.0 ± 0.0 ^{a}	-	< 5.0 ± 0.0 ^{a}	< 5.0 ± 0.0 ^{a}	-	< 5.0 ± 0.0 ^{a}	< 5.0 ± 0.0 ^{a}	-
Lactones															
γ-nonalactone	15.5 ± 0.7 ^{c}	10.5 ± 0.7 ^{c}	-32*	20.5 ± 0.7 ^{bc}	13.0 ± 1.4 ^{c}	-37	34.0 ± 2.8 ^{a}	68.0 ± 4.2 ^{a}	100*	18 ± 4.2 ^{bc}	19.0 ± 2.8 ^{c}	6	28.5 ± 3.5 ^{ab}	38.0 ± 1.4 ^{b}	33

Compound	Control			PS2			R2Δ			R29			FST1.7		
	FS	FAS	Δ	FS	FAS	Δ	FS	FAS	Δ	FS	FAS	Δ	FS	FAS	Δ
Furans															
2-Furfural	24.0 ± 0.0 [a]	25.5 ± 0.7 [d]	6	< 5.0 ± 0.0 [b]	65.0 ± 0.0 [b]	1200*	< 5.0 ± 0.0 [b]	46.0 ± 4.2 [c]	820*	< 5.0 ± 0.0 [b]	47.5 ± 3.5 [c]	850*	37.0 ± 8.5 [a]	118.0 ± 7.1 [a]	219
2-Acetylfuran	< 5.0 ± 0.0 [a]	< 5.0 ± 0.0 [a]	-	< 5.0 ± 0.0 [a]	< 5.0 ± 0.0 [a]	-	< 5.0 ± 0.0 [a]	< 5.0 ± 0.0 [a]	-	< 5.0 ± 0.0 [a]	< 5.0 ± 0.0 [a]	-	< 5.0 ± 0.0 [a]	< 5.0 ± 0.0 [a]	-
5-Methyl-Furfural	< 5.0 ± 0.0 [a]	< 5.0 ± 0.0 [a]	-	< 5.0 ± 0.0 [a]	< 5.0 ± 0.0 [a]	-	< 5.0 ± 0.0 [a]	< 5.0 ± 0.0 [a]	-	< 5.0 ± 0.0 [a]	< 5.0 ± 0.0 [a]	-	< 5.0 ± 0.0 [a]	< 5.0 ± 0.0 [a]	-
2-Propionylfuran	< 5.0 ± 0.0 [a]	< 5.0 ± 0.0 [a]	-	< 5.0 ± 0.0 [a]	< 5.0 ± 0.0 [a]	-	< 5.0 ± 0.0 [a]	< 5.0 ± 0.0 [a]	-	< 5.0 ± 0.0 [a]	< 5.0 ± 0.0 [a]	-	< 5.0 ± 0.0 [a]	< 5.0 ± 0.0 [a]	-
Total staling indicators	211.5	255		67.5	286.5		76	224.5		107.5	201		125.5	266	
Total oxygen indicators	167	214		42	193		36	96		84	129.5		54.5	105	
Total thermal indicators	39.5	36		20.5	78		34	114		18	66.5		65.5	156	

Each value was expressed as mean ± standard deviation analysed in duplicate from two independent fermentations.

[a-d] For each compound, a different superscript in each row denotes a significant difference at $P < 0.05$.

* For each compound and treatment, an asterisk (*) denotes a significant difference at $P < 0.05$.

7.5 Conclusion

The results indicated that strain-dependent physiological and metabolic features of the starter culture have a significant impact on the flavour-forming mechanisms during fermentation of malt-based substrate aimed at novel beverages. The control of physico-chemical parameters such as temperature, pH and nutrient composition, and optimisation of the processes employed, e.g. mashing, filtration or pasteurisation, are potential steering points for the final aroma and taste profile. The barley malt-based medium was able to support growth of LAB to varying extents and without nutrient supplementation. Of the four LAB tested, *Lb. plantarum* FST1.7 and *Lb. reuteri* R29 were able to accumulate the most lactic acid, with the latter strain releasing the highest amount of acetic acid as well, conveying "pungent" notes to the drink. The fermentable sugars as well as the nitrogen source were not the restricting factors on LAB growth, but other aspects such as pH level or lack of micronutrients could have been responsible for the decrease in growth in individual cases. Considering the specific preference for carbon and nitrogen source for each strain, it would be conceivable to modulate the sugar and amino acid (Chen et al., 1973) concentration and composition through optimisation of the mashing program with important effects on the final quality properties. Moreover, the availability of substrates for the Maillard reaction will have significant consequences on the final flavour profile, although extensive predictability of the aromatic results still remains an ambitious goal. The presence or absence of specific flavour-active compounds are often the result of highly interdependent biological and biochemical mechanisms (Boulton and Quain, 2001a). Fermentation resulted in better organoleptic stability during ageing and could improve the flavour complexity of malt-based beverages. The chemical analysis revealed the presence of less staling indicators in fresh fermented samples, but the same compounds generally increased during ageing to a greater extent compared to the unfermented control wort. In order to draw conclusions on the final quality and acceptability of the product, both sensorial and analytical stability of the flavour profile have to be approached simultaneously, since one does not determine the outcome of the other. It should be mentioned that metabolites traditionally associated with "off-flavour" in beer can be positively reconsidered when present in other cereal-based beverages, especially if combined with health or organic claims (Fernqvist and Ekelund, 2014). Nevertheless, complementary studies would be necessary in order to identify those flavour compounds relevant in lactic fermentation

of cereal malt-based substrates and complement them to the established flavour spectrum routinely analysed for beer.

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Chapter 8: Overall discussion and conclusion

8.1 Overall discussion and conclusion

The food industry is exploring alternative manufacturing and preservation methods that comply with the increasing consumer demand for clean-label technologies and minimally processed foods (Zink, 1997). Fermentation has long been used as a way to naturally improve, conserve and/or fortify a substrate, without the need for additives (Hugenholtz, 2013). The ability of lactic acid bacteria (LAB) to release a broad range of functional metabolites, together with their safe to consume status, provides the starting point for numerous applications in malting, brewing and for the development of novel, cereal-based beverages. A literature review was conducted as part of this thesis (**Chapter 2**). A variety of antimicrobial compounds released by LAB have previously been investigated and applied with success during cereal processing, e.g. sourdough fermentation or barley malting, to control spoilage microbiota (Axel et al., 2016a; Lowe and Arendt, 2004; Rouse and van Sinderen, 2008; Vaughan et al., 2005). The bioprotective effect of LAB has long been attributed to acidification by organic acids, but studies are increasingly reporting about the role played by compounds other than lactate and acetate and the importance of interactions between these different antimicrobial molecules (Axel et al., 2016b; Black et al., 2013; Oliveira et al., 2015). As the main metabolite released by LAB, lactic acid (LA) plays an important role for the adjustment of the pH during mashing or in the production of sour beers. LA can be obtained via lactic fermentation of mash, wort, or otherwise organic waste. Increasing the knowledge on the impact that strain, substrate, and fermentation conditions have on LA production can help brewers to increase purity and productivity. Apart from LA, LAB can also produce a wide range of flavour-active compounds. Lactic fermentation can therefore improve the rather poor organoleptic properties of raw cereal-based beverages. Very little research has been done on malt-based substrates. An understanding of the quality, quantity and stability of the flavour compounds produced during fermentation in this medium could accelerate their acceptance and commercialisation while providing a product with unique selling points for breweries.

In **Chapter 3**, a comparison of different LAB cultures (*Lb. plantarum* FST1.7, *Lb. brevis* R2Δ and *Lb. brevis* L1105) revealed that the type and amount of organic and phenolic acids released in wort are species- and strain-dependent. The antifungal activity against *Fusarium culmorum* macroconidia was primarily attributed to lactate and acetate, while phenolic acids were released at concentrations at which only weak synergies with the

low pH and organic acids could be detected. However, the very low MIC values calculated for some of these compounds, e.g. benzoic, ferulic and *p*-coumaric acid, confirmed their potential as effective antifungal compounds. The high variability of the MIC values amongst the phenolic compounds can be explained by the different pKa values (Lind et al., 2005; Oliveira et al., 2014), the presence of unsaturated side chains and the extent of ring substitution (Sánchez-Maldonado et al., 2011). These molecular features will ultimately dictate the tendency for trans-membrane diffusion into the fungal cytoplasm. Fermentation of substrates with high malt extract (12% (w/w)) encouraged production of both organic and phenolic acids, leading to considerable accumulation of LA, 3-phenyllactic acid and hydroferulic acid. The presence of more nutrient precursors (monosaccharides, amino acids, …), as well as a higher buffering capacities (BC), were likely to be beneficial for the increased release of phenolic acids. In this regard, BC should be carefully adjusted to a level that still allows low pH values to be reached after fermentation, in order to exploit the synergistic activity of antifungal acids. In addition, the utilisation of higher extract substrates should consider the presence of high residual nutrients after lactic fermentation, which could eventually encourage the growth of any spoilage microorganisms present, e.g. on the grain surface. On the other hand, when using diluted wort, longer fermentation times could partially compensate for the lower extract content.

This research contributed further knowledge about the mode of action of phenolic acids and the synergistic interaction that exists between them. The more effective antifungal activity of acetate compared to lactate encourages the screening for obligate heterofermentative starter cultures (Table 26). Strains possessing proteolytic activities could increase the pool of amino acids *in situ* to be used as a substrate for phenolic acid synthesis (Oliveira et al., 2014). To this end, studies have successfully shown that the addition of specific precursors could increase the concentration of derived phenolic compounds, e.g. production of 3-PLA from phenylpyruvic acid, during batch (Valerio et al., 2016) and fed-batch fermentations (Rodríguez et al., 2012). LAB could also be selected according to their ability to convert phenolic acids into derivatives that display lower MIC values, similarly to what was found for *Lb. plantarum* FST1.7. The ease of identifying acid-based antifungal compounds allows also for faster selection procedures during starter culture screening. However, acids represent only a fraction of the antimicrobial compounds released by LAB, as was confirmed during the spiking experiments. These showed that inhibitory compounds, e.g. cyclic dipeptides or

proteinaceous compounds (Dal Bello et al., 2007), other than carboxylic acids accounted for ca. 2/3 of the antifungal effect produced by FST1.7.

In a complex food matrix, the application of LAB starter cultures and their fermented spent media needs to be tailored according to the processing conditions (e.g. temperature, aeration, water content), initial fungal load, and the potential of LAB cross-contamination within the manufacturing premise. The antifungal strain *Lb. brevis* R2Δ was selected among other candidates because of its ability to grow at malting temperatures (ca. 14°C) (Appendix 4) and its hop sensitivity, enabling better control within a brewing environment. Different treatments based on varying the concentrations of LAB cells and/or spent wort media were applied on barley grains during pilot-scale malting (**Chapter 4**). The very high number of LAB cells (ca. 10 log) on the surface of a single grain confirmed that the application of R2Δ throughout steeping and germination was successful. The advantages of adding the bioprotective culture to the steeping water as opposed to spraying are 1) the earlier fungal inhibition, 2) the thorough grain coating and 3) the easier scale-up of the technology. Similar to the addition of pure organic acids, fermented worts decreased the acid-sensitive aerobic bacteria while increasing yeast population on the grain surface. The promotion of yeasts could be intentionally pursued as certain indigenous species, e.g. *Pichia anomala*, can contribute to bioprotection and enzymatic activity (Boivin and Malanda, 1997; Laitila et al., 2007). In contrast, the application of washed LAB cells did not influence the target microflora compared to the control, confirming that production of bacterial metabolites, rather than intercellular competition, was the primary determinant of inhibition (Haikara et al., 1993; Niku-Paavola et al., 1999). Nevertheless, the presence of LAB cells was decisive in reducing mycotoxin levels, as confirmed by the lower value of enniatins found when only cells were applied compared to the control. The best containment of *Fusarium* infection was achieved when fermented wort ingredients containing live LAB cells were added, reducing the number of infected kernels and the total amount of *F. culmorum* and *F. graminearum* DNA. A portion of the bacterial metabolites responsible for the antifungal effect were likely to be heat-labile, as suggested by the poor *Fusarium* reduction achieved when the fermented wort was pasteurised (Mauch et al., 2010). This is in contrast with the results found in Chapter 3, in which the antifungal activity for *Lb. brevis* R2Δ was explained solely by the organic acids and low pH. Barley grains treated with the most acidic treatment reduced malting losses and yielded a higher level of soluble extract and nitrogenous compounds. The

lower α-amylase activity of this malt did not negatively influence soluble extract content of the resulting worts. Reduced modification of treated malts can be otherwise compensated through adaptation of mashing procedures (Mauch et al., 2011). The advantages of pH values in the range of 5.2-5.4 (optimum for enzymatic activity) during mashing were not observed in this study. To reach these low pH levels, additional acidification of the grain surface has to be done after completed germination. Finally, no negative impact was detected throughout alcoholic fermentation for any of the treated malts.

Overall, the best results were obtained using a fermented wort solution that contained living LAB cells producing high levels of organic acids (as well as other, unidentified bacterial metabolites). On one hand, the acidic wort substrate alone effectively inhibited the initial fungal spread, while the presence of LAB cells was necessary for mycotoxin reduction. In addition, this treatment contributed to increased production yield while retaining the technological properties of the malt. Acidity levels must be carefully balanced to achieve the desired spoilage inhibition while allowing the correct synthesis and/or activation of enzymes in the germinating kernels.

Lactic acid fermentation is a low-cost, energy-efficient process that allows the production of LA from agricultural and/or industrial waste substrates (Wang et al., 2015). In the brewery, production of LA for use in brewing applications can be directly done using malt extract as a substrate. Batch fermentations of wort, however, are subjected to self-inhibition of LAB growth at increasing acidity levels. **Chapter 5** focussed on improving LA release in wort using three brewing-relevant LAB strains (*P. acidilactici* AB39, *Lb. amylovorus* FST2.11, and *Lb. plantarum* FST1.7) by modifying the BC of the substrate. Increase of the BC of wort would not only benefit organic acid production, but possibly that of other compounds also, e.g. phenolic acids (Chapter 3) or flavour compounds (Chapter 7). The extension of the proteolytic rest during mashing was chosen as a means of naturally raising the BC of wort. This was attributed to the release of smaller nitrogenous compounds possessing higher BC than their precursors (Cohn and Edsall, 1943). LA production, however, was not significantly higher in these worts compared to the control. Moreover, the extensive degradation of starch and/or dextrins during the longer mashing time played a role in the inferior LA production by the amylolytic strain FST2.11. To overcome this, unmalted starch material could be mixed with malt to restore the amount of long-chain polysaccharides. In addition, the

avoidance of vigorous wort boiling could prevent excessive coagulation of the nitrogen fraction, retaining more BC. A positive, linear correlation between FAN content and BC of the worts was established. To this end, strains possessing proteolytic activity could contribute to the degradation of wort proteins, which could increase the pool of buffering peptides. To simulate this, an external protease was added during mashing, and fermentation of the resulting worts allowed production of up to 24% more LA compared to the control wort. However, the low pH values found in the fermented substrates were once more responsible for inhibition of bacterial growth. To prevent this, citrate-based buffers were added to wort, which ultimately led to additional LA enrichment (up to 53%). Furthermore, the use of external buffers in a diluted wort (ca. 4.5 % (w/w)) achieved comparable LA concentration to that obtained in the unbuffered control wort (ca. 9 % (w/w)), and this could be regarded as a cost-effective option. In the case of worts containing external buffers, the depletion of essential nutrients and/or the accumulation of toxic compounds were likely the cause of growth cessation. In order to further exploit the potential of wort for LA production, BC should be matched with strain-dependent nutritional requirements. The inability to consume the maltose in the wort represented a hurdle to the growth of AB39 and FST1.7 strains. The preference for monosaccharides over disaccharides and/or trisaccharides, which are both abundant in wort, requires practices that modify sugar composition, e.g. the inclusion of exogenous enzymes such as amyloglucosidases, maltases or amylases, or the exploitation of the endogenous enzymes by applying special mashing procedures, e.g. Herrmann procedure (Appendix 6) (Herrmann et al., 2003). Depending on the strain-dependent metabolism (Mayo et al., 2010), yields of LA from different carbon sources varied significantly. Studies done in synthetic broth (MRS) containing single carbon sources, however, were not mirroring the preferential sugar consumption in wort. The presence of antagonistic mechanisms (e.g. carbon catabolite repression by glucose) during wort fermentation indicate that starter cultures should be screened directly in this substrate rather than in synthetic ones. The nature of the strain will also determine the process duration needed to maximise profitability of LA accumulation, with AB39 building between 73-80% (w/v) of the total LA within 24 h of fermentation, while FST2.11 only 63-67% (w/v). The complete utilisation of multiple amino acids was found for all strains after 48 h of fermentation. Depletion of amino acids is, however, not necessarily the reason for growth cessation, as strain-dependent metabolism allows the conversion of these compounds into each other (Christensen et al., 1999).

Nevertheless, fortification of wort could be done through addition of sources rich in organic nitrogen, such as yeast extract (Altaf et al., 2006) or malt sprouts (Laitila et al., 2004). In general, suitable strains should be screened primarily on the criteria of having homofermentative metabolism and good acid resistance (Table 26). The ability to consume sugars other than the monosaccharides, such as maltose and/or wort polysaccharides, is another desiderable metabolic trait.

Acidification of wort plays a primary role in the production of sour beers. In this regard, souring can be done at various time points throughout the brewing process. Ultimately, the decision of when to acidify will influence attributes of both technological and quality nature. As shown in **Chapter 6**, desired acidification (ca. 5-6 g/L LA) by *Lb. amylovorus* FST2.11 was achieved within 18 h of fermentation of mash and/or wort. In particular, for mash souring, a fast acidifying starter culture is preferable over slow acidifiers in order to quickly reach a pH < 4 and thus inhibit the growth of spoilage microorganisms still viable at mash out. Extract consumption as well as amylolytic and proteolytic degradation by FST2.11 produced mashes of lower viscosity, allowing for faster initial filtration. However, the high cell biomass in the soured mash could eventually deposit on the spent filter, reducing the efficiency of lautering later in the process (Tien and Ramarao, 2008). The hop sensitivity of the strain was prioritised in order to limit the potential for cross-contamination within the brewing premise. Otherwise, the use of hop-resistant cultures would be preferable when acidification is to be performed in hopped wort, e.g. at fermentation stage. Development of hop resistance can be induced in LAB by exposing them to subinhibitory concentrations of hop compounds (Simpson and Fernandez, 1992). This also means that the strain should be routinely refreshed from a stock culture according to the brewing schedule. Even though the low pH (< 3.5) after lactic fermentation impaired vigorous yeast growth in the soured substrates, all trials showed similar fermentation kinetics and reached final attenuations comparable to the unsoured control. The vast majority of the sugars (94.2 to 96.1%) and FAN (80.8 to 89.5%) were consumed by *S. cerevisiae* (Safale US-05) during alcoholic fermentation. Higher acidity levels of wort would have further impaired yeast vitality and metabolism (Table 17). As a way to prevent the disadvantages attributed to acid stress, yeast strains could be screened and selected according to their better adaptation to acidic conditions (Haitani et al., 2012). It is unclear if the proteolytic activity of FST2.11 was responsible for poorer foam retentions observed in the soured wort samples produced with this strain, or if malt melanoidins led to better foam stability as found in the soured mash

trials. To test these hypotheses, additional studies could be done to identify the molecules enriched in the foam tower. Lactic fermentation had a significant impact on the final organoleptic properties of sour beer. The high levels of acetaldehyde, diacetyl and acetoin found in the soured beers could have been caused by 1) active release by the bacterial culture, 2) reduced oxidative stability of beer at low pH, and 3) impaired re-absorption off these flavour compounds by the yeast. Longer fermentation and maturation times could eventually help to decrease the levels of these compounds, while ageing experiments could better elucidate the evolution of the organoleptic properties of these beers. Overall, the differences found in filterability, viscosity, haze, foam stability, flavours and organoleptic profiles confirm the far-reaching influence that the method of acidification can exert on important beer attributes.

Metabolites normally classified as "off-flavour" in conventional beer could be reconsidered as positive attributes when present in novel, cereal-based beverages, especially if the latter carry health-related claims (Fernqvist and Ekelund, 2014). The production of malt-based drinks constitutes an opportunity for breweries to exploit the consumer trend for functional beverages and overcome potential over-capacities. In this context, **Chapter 7** focussed on the fermentation of diluted wort (6°P) by four different LAB species (*Lb. plantarum* FST1.7*, Lb. reuteri* R29*, Lb. brevis* R2Δ*,* and *W. cibaria* MG7). The key parameter for future viability and success of cereal-based beverages is consumer acceptance, which is primarily driven by the flavour and the claimed nutritional aspects (Granato and Branco, 2010). In this study, special attention was placed on the differences in sensory attributes and flavour compounds produced in these beverages as well as their stability during ageing. The three obligate heterofermentative strains led to a higher ratio of acetic to lactic acid (AA:LA) compared to the facultative heterofermentative strain, *Lb. plantarum* FST1.7. The balance and proportion of these organic acids can play an important role on the final organoleptic properties, with acetic acid delivering a more pungent sourness and vinegar-like notes. In this regard, bacterial metabolism can be steered towards acetic acid production by regulating the addition of fructose and/or oxygen during fermentation (Kandler, 1983). Consumers have diverse tastes when considering sour products, but acidity is known to contribute to a refreshing and thirst-quenching effect (McEwan and Colwill, 1996), which can lower the palate fullness described for sweet, cereal-based beverages (Krahl et al., 2009). The bacterial consumption of specific sugars, while leaving others unfermented, can also influence the overall sweet taste of the final drink, with sweetness intensity following a descending

order from fructose, to glucose, and finally maltose. Customisation of organic acid concentrations through the ratio AA:LA and sugar/acidity could therefore be an asset for increasing acceptance (Nsogning Dongmo et al., 2016). All drinks were below the alcohol limit set for non-alcoholic beverages (< 0.5% (v/v) ethanol) (Kreisz et al., 2008), and the low pH complies with commercial examples of other fermented beverages, e.g. kombucha (pH between 3.0-3.4), although higher pH values (4.0-4.5) were also proposed as sensorially acceptable in cereal drinks (Angelov et al., 2006). Lactic fermentation of wort lowered the concentration of compounds responsible for "malty" notes (2-, 3-methylbutanal, 2-phenylacetaldehyde) and increased "buttery"-associated metabolites (diacetyl and acetoin). The low concentration of esters confirmed the limited ability of LAB to produce these molecules. The singular contribution of flavour compounds (isoamylacetate (fruity, banana), propan-1-ol (warm effect) and acetaldehyde (fruity, apple)) added to the flavour complexity of the malt-based beverages. Drinks reported with descriptors such as honey or apple scored better during sensory analysis than those carrying kwass or soya sauce. Ageing contributed to an increase in staling compounds in the fermented samples to a higher level than in the unfermented control. However, thermal deterioration indicators such as 2-furfural (caramel, bready) and γ-nonalactone (coconut, rancid) were not perceptible during sensory testing, and both drinks fermented with *Lb. reuteri* R29 and *Lb. plantarum* FST1.7 scored better than the aged, unfermented control. The contradiction between sensory and analytical results advocates for further studies aimed at identifying the key flavour compounds in lactic fermentation of cereal (malt)-based substrates. Ultimately, acceptance of these beverages will rely on the combination of intrinsic factors, such as flavour compounds, organic acid composition, and sugar content, with extrinsic factors, such as *natural*, *free-from* and *good-for-you* claims.

Overall, this thesis showed that LAB can ferment wort to produce a food-grade and readily available substrate that can serve multiple functions, such as a "green", antifungal ingredient during malting, a supply of organic acids for brewing operations and sour brewing, and a base for novel (non-alcoholic) beverages. Depending on the desired application, the screening process should evaluate bacterial strains with multiple, function-specific attributes in order to exploit this combination of strain and substrate to their fullest potential (Table 26).

Table 26. Main traits of LAB cultures to be considered when screened for different applications.

Bacterial traits	**Technological consequences**
Bioprotection of grains during malting	**Chapter 3 / 4**
Obligate heterofermentative	Release of acetate (MIC (acetate) < MIC (lactate))
Antifungal activity *in vitro*	Presence of other antifungal compounds (phenolics, proteinaceous compounds,…)
Acid resistance	Higher yields of acids
Hop sensitivity	Reduce spoilage potential in brewing premises
Multiple enzymatic activities	Improve grain modification
Specific metabolism	Ability to convert phenolic acids (high to low MIC)
Lactic acid hyperproduction	**Chapter 5**
Obligate homofermentative	Release of mostly lactic acid and limited by-products
Maltose / Polysaccharides metabolism	Provide energy after depletion of monosaccharides
High yields from sugar unit	Channelled production of LA
Acid / extract resistance	Ability to ferment under high acid / extract conditions
Hop sensitivity	Reduce spoilage potential in brewing premises
Multiple enzymatic activities	Utilisation of polysaccharides and polypeptides
Minor auxotrophies	Limited need for AA, minerals, vitamins
Flavour compounds	Release of average to minimal off-flavours
Specific metabolism	Conversion of AA to ones with higher BC
Sour beer production	**Chapter 6**
High acid yields	Leave high amounts of sugar for yeasts
Bioactive compounds	Release of zinc and vitamins B
Alcohol resistance	Improved viability during co-fermentation
Extract resistance	Acidification during high-gravity brewing
Hop sensitivity / resistance	Flexibility in hopping
Sensitivity to antibiotic / resistance to phage	Improved safe-to-use in industrial premises
Enzymatic activity	Amylolysis (faster lautering/filtration, less haze), proteolysis (AA as nutrients, faster filtration, better trub building)
Flavour compounds	Minimal off-flavours
Specific metabolism	Inability to induce premature yeast flocculation (42)
Development of novel beverages	**Chapter 7**
Homo-/Heterofermentative	Desired lactic to acetic ratio, limited alcohol production
Probiotic	Ability to retain viability in cereal media, adherence to intestinal mucosa, acid and bile tolerance

Exopolysaccharides producer	Release of prebiotics, improve mouthfeel
Polyols	Decrease calories intake while maintaining sweetness
Flavours	Minimal off-flavours
Enzymatic activity	Modulation of viscosity (amylolysis)

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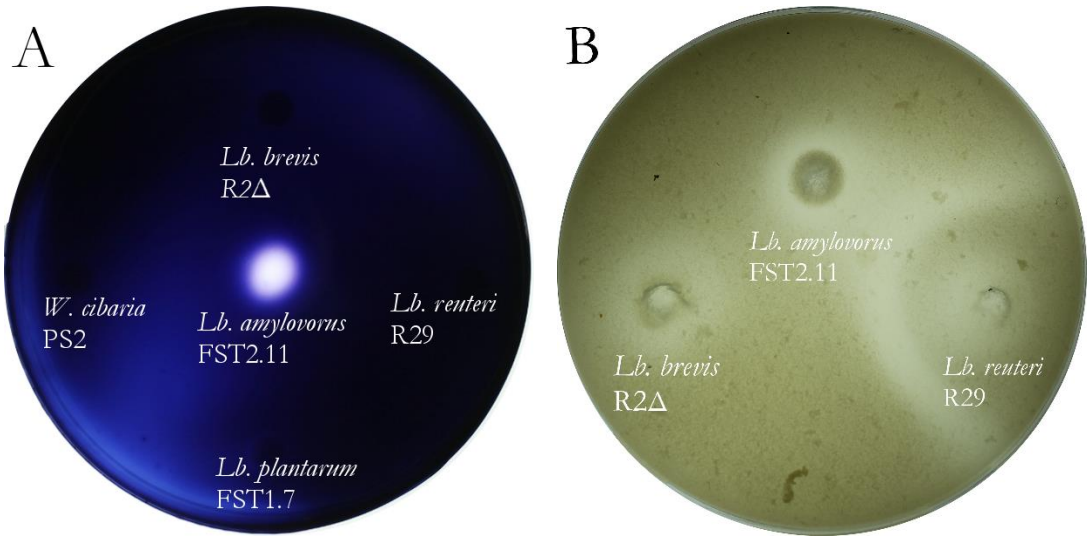
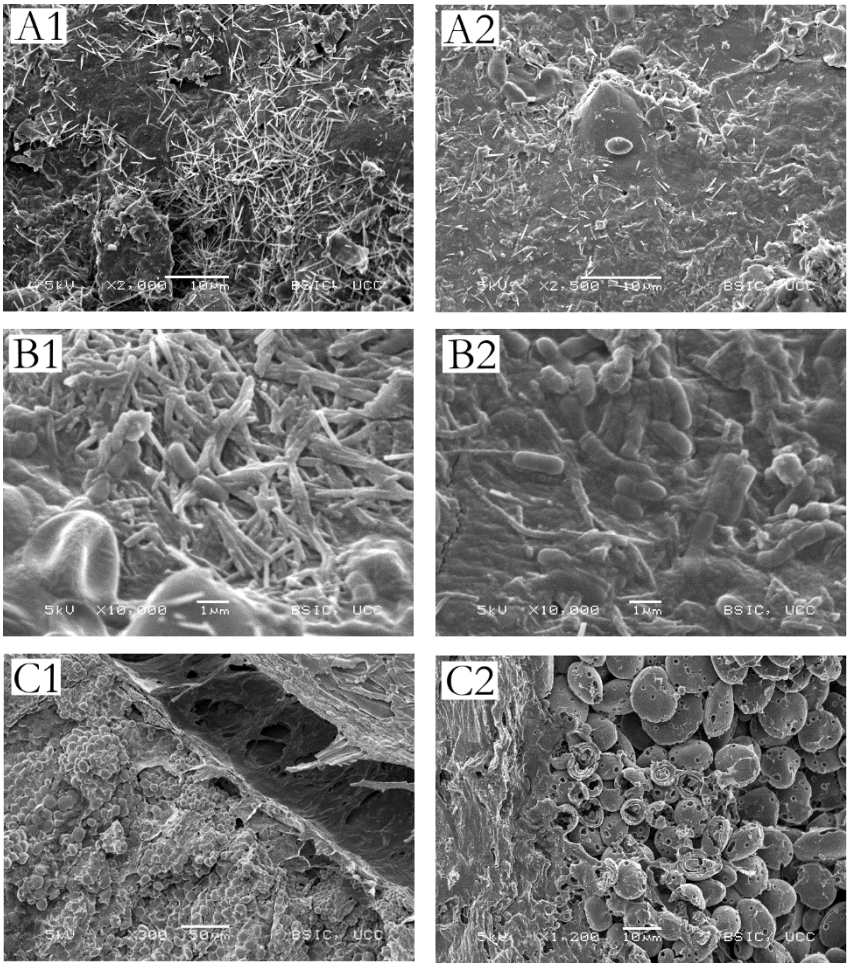
Appendix



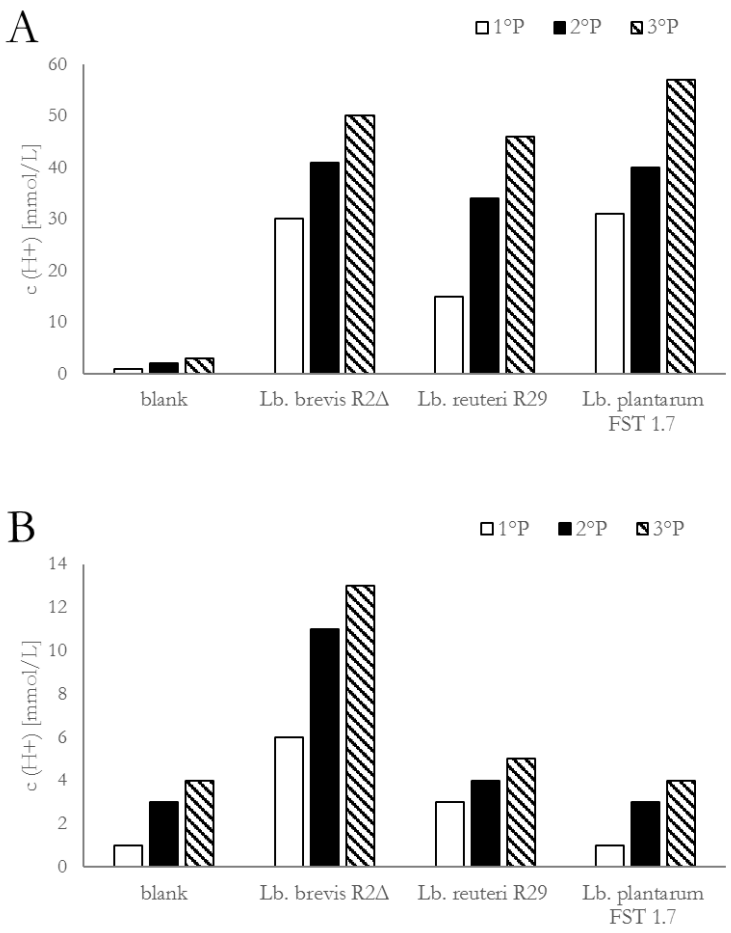
Appendix 1. (A) Amylolytic and (B) proteolytic activities tested on starch and milk agar plates, respectively, for different LAB strains.



Appendix 2. Scanning electron micrographs from malting of barley grains. Surface pictures showing microbiota before kilning on C-T (× 2,000) (A1) and FW (× 2,500) (A2); microbiota on LAB-T (× 10,500) (B1) and on FW (× 10,000) (B2) after kilning; starch in endosperm of C-T before (× 300) (C1) and after malting (× 1,200) (C2).

Appendix 3. Limit of detection (LOD) for mycotoxin analysed in Chapter 4.

Analyte	Limit of Detection (µg/kg)
15-Acetyl-Deoxynivalenol (15-ADON)	62.5 – 3000
3-Acetyl-Deoxynivalenol (3-ADON)	62.5 - 3000
Aflatoxin B1 (AFB1)	1 - 12
Aflatoxin B2 (AFB2)	1 - 12
Aflatoxin G1 (AFG1)	0.5 - 12
Aflatoxin G2 (AFG2)	1 - 12
Agroclavin (A-CLAV)	12.5 – 600
Alternariol (ALTER)	5 - 240
Alternariol-methyl-ether (ALTER-CH3)	10 – 240
Andrastin A (AND-A)	12.5 – 600
Beauvericin (BEA)	50 – 1200
Deoxynivalenol (DON)	62.5 - 3000
Deoxynivalenol-3-Glucoside (D3G)	12.5 - 300
Diacetoxyscirpenol (DAS)	2.5 – 120
Enniatin A (ENA)	25 - 600
Enniatin A1 (ENA1)	50 – 600
Enniatin B (ENB)	20 – 240
Enniatin B1(ENB1)	20 – 240
Fumonisin B1 (FB1)	25 - 1200
Fumonisin B2 (FB2)	25 – 1200
Fumonisin B3	12.5 - 600
Fusarenon-X (FUS-X)	20 – 240
Gliotoxin (GLIO)	12.5 - 600
HT-2 toxin (HT-2)	10 – 120
Mycophenolic Acid (MPA)	10 – 240
Neosolaniol (NEO)	12.5 – 600
Nivalenol (NIV)	62.5 - 3000
Ochratoxin A (OTA)	not reportable
Ochratoxin alpha	1.5 - 36
Ochratoxin B (OTB)	0.375 - 18
Patulin (Pat)	12.5 - 150
Roquefortine C (ROQ-C)	5 – 240
Roquefortine E (ROQ-E)	not reportable
Sterigmatocistin (STER)	5 – 120
T-2 toxin (T-2)	5 - 120
T-2 Triol (T2-3OH)	20 - 240
T-2-Glucoside (T2G)	10 – 240
Tentoxin (TEN)	12.5 – 600
Territrem B (TERR-B)	12.5 – 600
Wortmannin (WORT)	12.5 - 600
Zearalenone (ZEA)	6.25 – 150

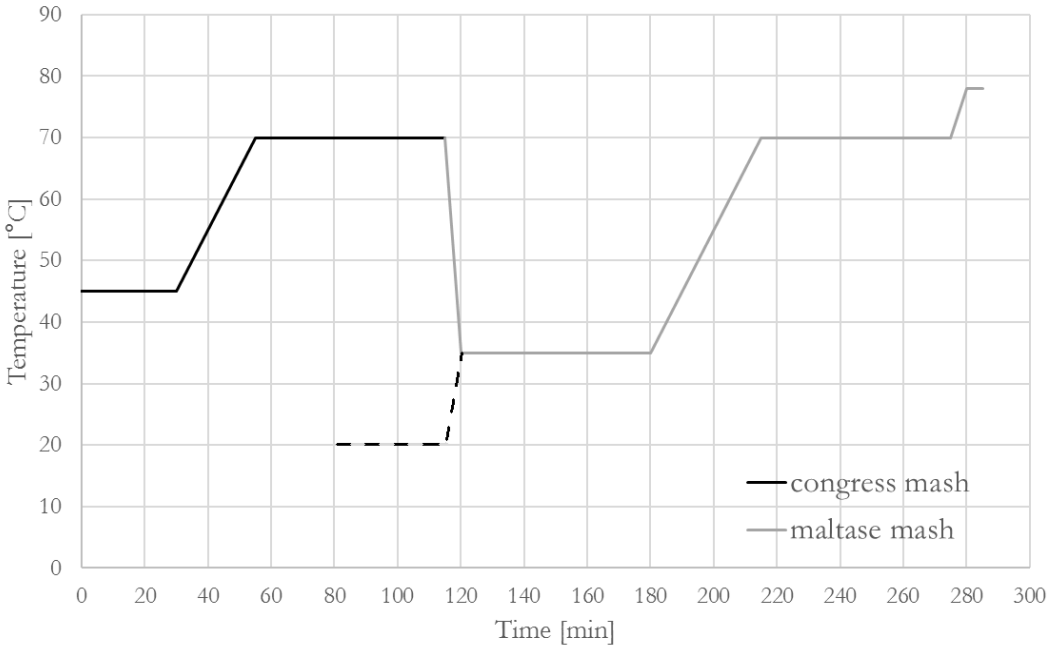


Appendix 4. Acidification pre-trials for *Lb. brevis* R2Δ, *Lb. reuteri* R29 and *Lb. plantarum* FST1.7 done in diluted wort (1, 2 and 3 % (w/w)) at optimal temperature (30°C) (A) and at malting temperatures (14°C) (B).

Appendix 5. Lactic acid production by strains from UCCs Food Science culture collection after fermentation for 48 h in Congress wort.

Species		**Lactic acid production**
Lactobacillus amylovorus	AB32, AB36, FST2.11	+++
Lactobacillus plantarum	FST1.7	+++
Pediococcus acidilactici	AB39	++
Lactobacillus manihotivorans	DSM13343	++
Lactobacillus rhamnosus	C7, C8, C9	+
Lactobacillus delbrueckii	WLP677, UCC5.1	+
Pediococcus pentosaceus	E6	+
Lactobacillus amylolyticus	FST3.5	+

+++ : > 5 g/L; ++ : 3–5 g/;, +: 1–3 g/L



Appendix 6. Mashing regime benefitting of endogeneous maltase (according to the Herrmann procedure).

Publications and presentations

First author publications

Peyer L.C., Bellut, K., Lynch, K.M., Zarnkow, M., Jacob, F. De Schutter, D.P., and Arendt, E.K. (2016). Impact of buffering capacity on the acidification of wort by brewing-relevant lactic acid bacteria. *Journal of the Institute of Brewing. Accepted*

Peyer L.C., Zarnkow, M., Jacob, F., De Schutter, D.P., and Arendt, E.K. (2016). Sour brewing: impact of *Lactobacillus amylovorus* FST2.11 on technological and quality attributes of acid beers. *American Society of Brewing Chemists Journal. Accepted*

Peyer L.C., de Kruijf, M., O'Mahony, J., DeColli, L., Danaher, M., Zarnkow, M., Jacob, F. and Arendt, E.K. (2016). *Lactobacillus brevis* R2Δ as starter culture to improve biological and technological qualities of barley malt. *Journal of European Food Research and Technology*. DOI 10.1007/s00217-017-2847-9 *(In press*)

Peyer, L.C., Zannini, E., Arendt, E.K. (2016). Lactic acid bacteria as sensory biomodulators for fermented cereal-based beverages. *Trends in Food Science & Technology*. 54, 17–25.

Peyer, L.C., Axel, C., Lynch, K.M., Zannini, E., Jacob, F., Arendt, E.K. (2016). Inhibition of *Fusarium culmorum* by carboxylic acids released from lactic acid bacteria in a barley malt substrate. *Food Control*. 69, 227-236

Peyer, L.C., Zannini, E., Jacob, F., Arendt, E.K. (2015). Growth study, metabolite development and organoleptic profile of a malt-based substrate fermented by lactic acid bacteria. *American Society of Brewing Chemists Journal*. 73(4), 303–313.

Other publications

Axel, C., Brosnan, B., Zannini, E., Peyer, L.C., Furey, A., Coffey, A., Arendt, E.K. (2015). Antifungal activity of three different *Lactobacillus* species and their production of acid-based antifungal compounds in wheat sourdough. *Applied Microbiology and Biotechnology*, 100(4):1701-11

Oral presentations

Peyer L.C., de Kruijf, M., O'Mahony, J., DeColli, L., Danaher, M., Zarnkow, M., Jacob, F. and Arendt, E. (2016). Different application methods of *Lactobacillus brevis* R2Δ and effects on malt and beer parameters. Young Scientists Symposium on Malting, Brewing and Distilling, Chico, California, USA, April 2016

Peyer, L.C. and Arendt, E. (2014). Characterisation of antifungal lactic acid bacteria and their application in malting and brewing. Young Scientists Symposium on Malting, Brewing and Distilling, Ghent, Belgium, October 2014

Peyer, L.C., Oliveira, P., Zannini, E., Jacob, F., Coffey, A. and Arendt, E. (2014). Isolation and characterisation of lactic acid bacteria with anti-fungal properties and their application in malting and brewing. 11th International Symposium on Lactic Acid Bacteria, Egmond Aan Zee, The Netherlands, August 2014

Poster presentations

Peyer L.C., Zarnkow, M., Jacob, F. and Arendt, E. (2017). Sour brewing: A comparison of acidification methods in the brewing process on wort and beer qualities. 36th Congress of the European Brewery Convention, Ljubljana, Slovenia, May 2017

Peyer L.C., de Kruijf, M., O'Mahony, J., DeColli, L., Danaher, M., Zarnkow, M., Jacob, F. and Arendt, E. (2016). Application of *Lactobacillus brevis* R2Δ as an antifungal culture to improve safety and quality of malt and beer. World Congress of Food Science and Technology, Dublin, Ireland, August 2016

Peyer, L.C., Axel, C., Zarnkow, M., Zannini, E., Jacob, F. and Arendt, E. (2015). Characterisation of antifungal lactic acid bacteria (LAB) and their application during malting. 35th Congress of the European Brewery Convention, Porto, Portugal, May 2015